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PRINCIPAL INVESTIGATOR: Dr. Olutosin Remi Idowu

CONTRACTING ORGANIZATION: Dr. Olutosin Remi Idowu
                            Silver Spring, Maryland 20910

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Dr. Olutosin Remi Idowu

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13. ABSTRACT (Maximum 200)

The following is a report on our study to identify the metabolites of artelinic, an artemisinin (qinghaosu) derivative being developed for uncomplicated malaria.

Identification of the metabolites of artelinic acid and other artemisinin analogues has been problematic because of the unavailability of standards of the putative metabolites. The following report presents our attempts to prepare the putative metabolites of artelinic acd by reacting artelinic acid with chemical systems that may simulate the activity of the cytochrome P-450 system.

Standards of possible metabolites of artelinic acid were produced by the reaction of artelinic acid with complexes of iron(II) and manganese(II) that may simulate cytochrome P-450 catalysed metabolism of xenobiotics. The standards were used to identify metabolites formed on incubation of artelinic acid with human liver microsomes.

The metabolism of artelinic acid by human liver microsomes involved isomerization/rearrangement involving the endoperoxide group, C-hydroxylation and debenzylation to dihydroqinghaosu (DQHS), an important active metabolite. Debenzylation of artelinic acid to DQHS is mediated by CYP3A4.

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FOREWORD

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<u>Study Title</u>

A Study of The Biomimetic Metabolism of Artelinic Acid By Chemical Cytochrome P-450 Model Systems

Investigator: _____
Olutosin Remi Idowu, Ph.D.

Acknowledgment

Foreword

The following is a report on our study to prepare standards of the putative metabolites of artelinic acid, an artemisinin (qinghaosu) derivative being developed as an oral treatment for malaria caused by multi-drug resistant *P. falciparum*. This study is part of the work going on at the Walter Reed Army Institute of Research (WRAIR) with the goal of developing a new generation of antimalarial drugs based on qinghaosu (artemisinin), the active principle of the Chinese medicinal plant, *Artemisia annua*.

Identification of the metabolites of artelinic acid and other artemisinin analogues has been problematic because standards of the putative metabolites are difficult and expensive to synthesize and are, therefore, not usually available   The following report presents our attempts to prepare standards of putative metabolites of artelinic acid by its reaction with chemical systems that may simulate the action of the cytochrome P-450 enzymes.

Biomimetic isomerization of artelinic acid to the furan acetate isomer and 3-hydroxydeoxyartelinic acid, and its deoxygenation to deoxyartelinic acid, were readily effected by its reaction with complexes of iron(II) in an appropriate solvent.

Reaction conditions for the biomimetic hydroxylation of artelinic acid by iron(II) complexes were extensively studied. Mono- and dihydroxylation of artelinic acid were achieved by the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline analogs substituted in the 5-position with electron-attracting groups, and in the presence of pyridine and ethanol (or methanol.)

The knowledge gained through this study should be of value in the development of other artemisinin drugs as well as other antiparasitic agents that may be of interest to the Walter Reed Army Institute of Research.

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3.4. DISCUSSION

1. INTRODUCTION

1.1. General

Qinghaosu (QHS) (also known as artemisinin), a sesquiterpene lactone endoperoxide, is the clinically active antimalarial principle isolated from the Chinese medicinal herb, Qinghao or *Artemisia annua* L (1, 2).

For nearly fifteen years the Walter Reed Army Institute of Research (WRAIR), working in collaboration with the Steering Committee of the Scientific Working Group on Malaria of the World Health Organization (SWG-CHEMAL), has actively pursued the development of new anti-malarial agents based on the lead provided by qinghaosu (QHS).

The development of semi-synthetic derivatives of QHS with improved physico-chemical and/or pharmacological properties has involved the reduction of QHS to the lactol, dihydroqinghaosu (dihydroartemisinin; Figure 1, DQHS), and subsequent preparation of the ether (or ester) derivatives of DQHS which are soluble either in water or oils and may be administered parenterally in the treatment of cerebral malaria

The oil-soluble derivatives of DQHS of interest are artemether (AM) and arteether (AE), which are the methyl and ethyl ethers of DQHS respectively.

The water-soluble derivatives of DQHS of clinical interest are artesunate (AS) the hemisuccinate ester of DQHS and artelinic acid (AL) the 4-carboxybenzyl ether of DQHS. The development of artelinic acid as an oral treatment for uncomplicated malaria caused by multidrug resistant *P. falciparum* is a top priority of the US Army Medical Research and Materiel Commmand (USAMRMC).

Artelinic acid is a semi-synthetic derivative of QHS which was prepared in this institute and the U.S. Army has exclusive rights and patents on artelinic acid. Artelinic acid has been reported to be effective against chloroquine resistant *Plasmodium falciparum* and has superior *in vivo* activity against *P. berghei*. When prepared as a solution in potasium carbonate this compound has been reported to be stable to hydrolysis (3). This is a desirable advantage over sodium artesunate, the only other water-soluble derivative of QHS, which has been extensively studied in China and Southeast Asia for the treatment of cerebral malaria. Sodium artelinate also has the

advantage of being the only water-soluble derivative of QHS which can be administered by iv infusion (4)   Artelinic acid is, therefore a promising future drug for the treatment of cerebral malaria. Preliminary studies at this institute have also shown that artelinic acid has other advantages over the other QHS derivatives including its high plasma levels following iv or im or oral administration, its relatively long elimination half life and high bioavailability in rat and dog.

However, artelinic acid seems to undergo less conversion to dihydroqinghaosu (DQHS), the active metabolite common to the QHS derivatives.   If, as has been suggested, both the anti-malarial activity (5) and toxicity (6) of the QHS derivatives are largely due to DQHS, the metabolism of artelinic acid will be expected to influence its anti-malarial activity and toxicity.   It is, therefore, necessary to understand the metabolism of artelinic acid.

Furthermore, it was thought that the metabolism of artelinic acid might be quite different from that of other QHS derivatives because artelinic acid possesses a benzyl group, a potential target of metabolising enzymes, which is absent in other QHS derivatives.   A study of the *in vitro* metabolism of artelinic acid is one of the neccesary steps towards the goal of the U.S. Army to develop artelinic acid as an oral treatment for uncomplicated malarial.

1.2. Identification of the *in vitro* Metabolites of Artelinic Acid

The metabolism of artemisinin analogues has been reviewed (7), and generally involves rearrangement and deoxygenation of the endoperoxide group, and hydroxylation on the A- and C- rings of the molecule.   Possible sites of hydroxylation are the 1,2,3,8,9,10 and 14 positions of the molecule (see Figure 1)   Thus the metabolic hydroxylation of these compounds may, in theory, give rise to isomeric hydroxy compounds which would be difficult to distinguish in the absence of authentic standards.

Indeed, the essential problem in studies of the metabolism of artemisinin and its derivatives has been the non-availability of authentic standards of the metabolites to be used for unambiguous identification of the microsomal or *in vivo* metabolites of these compounds. This problem arises because total synthesis of the artemisinin skeleton is involved and expensive.   Because other aspects of the metabolism studies depend on the availability of authentic standards of metabolites, our study has involved attempts to establish methods for making the metabolites of artelinic acid.

So far, the only known approach to making the metabolites of an artemisinin analogue is through fermentation of the compound by fungi. The fungal fermentation method is unpredictable in terms of the right microorganism to use in order to closely mimic the mammalian metabolism of the compound. Furthermore available reports have also shown that fungal metabolism of these compounds may not correlate well with the mammalian metabolism, with a particular fungus producing only one or two metabolites which are also mammalian metabolites. This has meant that different fungi had to be sought to produce different mammaliam metabolites. Finding the right microorganism, therefore, requires some trial and error effort. For example, in a study of the microbial metabolism of artemisinin itself, a total of 33 microorganisms had to be screened only to be able to select two that would produce two mammalian metabolites (8). The low yields of metabolites obtained is another drawback of this approach. Of the artemisinin analogues, only the fungal metabolism of arteether has been reported (9, 10, 11, 12).

We, therefore, sought a more efficient and less time-consuming method of making the metabolites of the artemisinin compounds through the reaction of the compounds with chemical models of cytochrome P-450.

1.3. Preparation of Metabolites of Artelinic Acid by Reaction of Artelinic Acid with Chemical Models of Cytochrome P-450

The catalytic mechanism of the cytochrome P-450 enzymes has been extensively studied and many chemical models of P-450 have been developed to elucidate the functions of these enzymes. In these studies, however, only very simple compounds such as cyclohexane, styrene, adamantane, aniline, toluidine, etc have been used as substrates. There have been very few reports on the application of chemical models of the P-450 enzymes to study the metabolism of actual drugs. Application of model systems to study drug metabolism has many attractive advantages. The most important of these are the simplicity of procedure and the formation and isolation of metabolite candidates in sufficient amounts to be used to identify the real *in vivo* metabolites. Application of model systems to study drug metabolism may also cut down on the use of experimental animals.

We studied the reaction of artelinic acid, with a number of P-450 model systems in order to isolate and characterize standards of metabolites which could be used for unambiguous identification of *in vitro* and *in vivo* metabolites of artelinic acid. Part of our results on

the biomimetic metabolism of artelinic acid by chemical models of cytchrome P-450 have been published (13). A copy of the journal article is included with this report as Appendix 1.

1.3.1. Pathways of Artelinic Acid Metabolism and their Simulation with Chemical Models of Cytochrome P-450

From studies with other artemisinin derivatives, the metabolism of artelinic acid would be expected to involve three chemical pathways, apart from the predictable possible debenzylation to DQHS. These three likely pathways are illustrated in Figure 2. To obtain standards of metabolites of artelinic acid, chemical model systems of cytochrome P-450 are required that would bring about the transformations of artelinic acid illustrated in Figure 2.

Rearrangement of the endoperoxide group without the loss of any of its oxygen atoms would result in the isomerization of artelinic acid to the furano acetate isomer (I, Figure 2) and the 3-hydroxydeoxyartelinic acid isomer (II, Figure 2). Deoxygenation of the endoperoxide goup would give rise to deoxyartelinic acid. The other likely metabolic pathway would involve C-hydroxylation of the molecule without the destruction of the endoperoxide group.

The rest of this report is divided into two sections, based on our attempts to chemically simulate these two pathways of artelinic acid metabolism. Section I of the report describes the reactions that yielded products formed by the rearrangement, or isomerization, of artelinic acid. Section II of the report describes reactions with which we sought to achieve the biomimetic hydroxylation of artelinic acid.

2.   SECTION I

2.1   BIOMIMETIC ISOMERIZATION/REARRANGEMENT OF ARTELINIC ACID BY REACTION WITH COMPLEXES OF IRON(II)

2.1.1 MATERIALS AND METHODS

Chemicals

All reagents and chemicals were obtained from Aldrich Chemical Co (Milwakee, USA) except artelinic acid and sodium artelinate which were which from the Walter Reed Inventory.

Instrumentation

2.1.1.1. Isolation of Reaction Products

Preparative HPLC was performed on a Waters liquid chromatography system consisting of two Waters model 510 solvent delivery units, a U6K injector and a Waters model 440 UV detector set at 254 nm.   A Waters μBondapak C_{18} preparative column (7.8 mm x 300 mm; 10 mm) was used with a mobile phase consisting of 0.1M ammonium acetate buffer (pH 4.5) and acetonitrile.   Elution was done with either a stepwise gradient of 70:30 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 56 min and then changed to a 50:50 ratio at 86 min, and held at this ratio for a further 20 min (flow rate 2.0 ml/min), or 80:20 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 40 min, changed to a 70:30 (v/v) ratio for 46 min, and then changed to a 50:50 ratio where is held for a further 30 min.   Acetonitrile was removed from the collected fractions with a stream of nitrogen and the fractions were then extracted with ethyl acetate (3 ml) by shaking on a vortex mixer for 2 min.   The ethyl acetate extracts were dried with anhydrous sodium sulphate and evaporated with a stream of nitrogen.   The residues were crystallised from a mixture of diethyl ether and hexane.   The isolated products were again re-analysed by HPLC-MS as described below, to establish their purity.

The reaction products were also separated by repeated preparative thin layer chromatography on 10 x 20 cm preparative silica gel GF plates, with dichloromethane:ethyl acetate (50/50, v/v) as solvent.   The substances in the bands were extracted with 20 %

methanol in ethyl acetate, the solvent removed, and the residue crystallised from ether-hexane as describe above.

2.1.1.2. Characterization of Reaction Products

Mass spectrometric identification of the reaction products was performed using a HPLC-MS system consisting of a Hewlett Packard 1090 Liquid Chromatograph System linked with a Hewlett Packard HP 5989A Mass Spectrometer via a Hewlett Packard thermospray interface. A μBondapak C_{18} column (2.1 mm x 100 mm; 5 m) was used, with a mobile phase consisting of 0.1M ammonium acetate (pH 4.5) and acetonitrile. Elution was done with either of two linear gradients: 95:5 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 30:70 ratio at 60 min, and held at this ratio for a further 20 min (flow rate 0.4 ml/min), or 90:10 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 60:40 ratio at 60 min, and held at this ratio for a further 30 min (flow rate 0.4 ml/min). The thermospray interface was operated in the "fragmenter on" mode at a vaporiser temperature of 85-96°C (or 87-105°C) and a source temperature of 220°C.

^{1}H- and ^{13}C-nmr spectra were obtained on a Brucker AC 300 Spectrometer, using $CDCl_3$ as solvent and Me_4Si as internal standard.

Melting points (uncorrected) were determined on a Thomas Hoover Unimelt^R capillary melting point apparatus.

2.1.1.3 Reaction of Artelinic Acid with the Udenfriend System (Fe^{2+}-Ascorbic acid-O_2)

Ferrous sulphate heptahydrate (1.22 g; 4.388 mmole) was dissolved in 100 ml of water followed by disodium-EDTA dihydrate (3.25 g; 8.732 mmole). After stirring the mixture for about 15 min, sodium artelinate hydrate (1 g: 2,183 mmole) was added followed by ascorbic acid (15.38 g; 87.326 mmole). The mixture was stirred vigorously for 15 h on magnetic stirrer. The mixture was then extracted thrice with 60 ml of ethyl acetate, by stirring on a magnetic stirrer for 1 h. The solvent was removed with a stream of nitrogen and an oil was obtained. The oil was analysed by HPLC-thermospray mass spectrometry and fractionated by repeated preparative TLC of by preparative HPLC.

2.1.1.4. Reaction of Sodium Artelinate with Hemin and Cysteine in Water

Sodium methoxide (63 mg; 1.16 mmole) was dissolved in 10 ml of water in a 250 ml RBF followed by 140 mg (1.16 mmole) of cysteine and 150 mg (0.23 mmole) of hemin. After stirring for 5 min, sodium artelinate (500 mg; 1.092 mmole) and a further 10 ml of water were added. The mixture was stirred at room temperature for 15 h. and then centrifuged at 3000 rpm for 15 min. The dark precipitate was extracted with ether to obtain an off-white solid which was found to be essentially unchanged artelinic acid. The supernatant was extracted with ethyl acetate (2 x 30 ml) by stirring on magnetic stirrer for 45 min. After each extraction, the mixture was centrifuged at 3000 rpm for 15 min. The ethyl acetate was removed and evaporated with a stream of nitrogen. An oil (170 mg) was obtained. and was analysed by LC-MS. The oil was chromatographed by preparative TLC on silica gel GF plates with dichloromethane:ethyl acetate (60:40, v/v) as solvent.

2.1.1.5. Reaction of Sodium Artelinate with Hemin and Glutathione in Water

The above procedure was reapeated, using 350 mg (1.14 mmol) of glutathione in place of cysteine.

2.1.1.6. Reaction of Artelinic Acid with Hemin and Cysteine in aqueous Acetone

Hemin (780 mg; 1.2 mmol) was dissolved in 50 ml of acetone. While stirring vigorously with a magnetic stirrer, a solution of 300 mg (2.5 mmol) of cysteine in 10 ml of water was added, followed by 250 mg (0.6 mmol) of artelinic acid. The mixture was stirred at room temperature for 18 h. The acetone was removed with a stream of nitrogen. The residue was stirred with 100 ml of water for 20 min and centrifuged at 3000 rpm for 15 min. The supernatant was extracted with 2 x 40 ml of ethyl acetate for 30 min while the precipitate was extracted with 4 x 30 ml of diethyl ether by shaking on a vortex mixer for 3 min. On evaporating the solvent both the ethyl acetate and the ether extracts yielded a dark brown, resinous oil, with more of the material being obtained from the ether extract of the precipitate. For initial purification, the resinous oil was

chromatographed on preparative TLC plates with ethyl acetate:dichloromethane (30:70, v/v) as solvent. The resinous material remained at the origin and the main band (rf about 0.3) was scraped and extracted with a 20:80 (v/v) methanol:ethyl acetate mixture yielding a pale yellow solid. The solid was analysed by LC-MS, and then chromatographed by preparative HPLC.

2.1.1.7. Reaction of Artelinic Acid with Hemin and Glutathione in aqueous Acetone

The above procedure was reapeated, using 740 mg (2.4 mmol) of glutathione in place of cysteine. A dark brown resinous material was also obtained as the reaction product on ether extraction of the precipitate as described above. Only traces of this reaction product was obtained from the ethyl acetate extraction of the supernatant. The initial clean-up of the resinous material with preparative TLC, recovery of a pale yellow solid and subsequent LC-MS analysis and preparative HPLC of the solid were also as described above for the reaction of artelinic acid with hemin and cysteine in acetone

2.1.1.8. Reaction of Artelinic Acid with Hemin and Thiosalicylic Acid in Acetone

Thiosalicylic acid (500 mg; 3.24 mmol) was dissolved in 50 ml of acetone, followed by hemin (936 mg; 1,44 mmol) and artelinic acid (300 mg; 0.72 mmol). The mixture was stirred for 18 h and subsequently processed as described above for the reaction of artelinic acid with hemin and cysteine. As with the preceeding reactions a dark brown resinous oil was obtained, mostly from the ether extraction of the precipitate formed upon centrifugation of the reaction mixture as described above. Preparative TLC of the crude material to obtain a pale yellow solid and subsequent LC-MS analysis and preparative HPLC of the solid were also as described above.

2.1.1.9. Reaction of sodium artelinate with Iron(II) sulphate and Thiosalicylic Acid in Water

Thiosalicylic acid (850 mg; 5.513 mmol) was mixed with 20 ml of water followed by sodium methoxide (80 mg; 1.4812 mmol) and iron (II) sulphate heptahydrate (760 mg; 2.734 mmol) and sodium artelinate hydrate (500 mg; 1.092 mmole). The mixture was stirred for 15 h and the centrifuged at 3000 rpm for 15 min. The clear supernatant (pH 5) was extracted with ethyl acetate (2 x 30 ml) by stirring on magnetic stirrer for 45 min. After each extraction,

remove the ethyl acetate, dry with anhydrous sodium sulphate and evaporate with a stream of nitrogen. The residue was chromatographed by preparative TLC on silica gel GF plates with dichloromethane:ethyl acetate (50:50, v/v) as solvent, and also by preparative HPLC.

2.1.1.10.   Reaction of Artelinic Acid with Iron(II) sulphate and Thiosalicylic Acid in Acetone.

Iron(II) sulphate (831.4 mg 2.99 mmol) was dissolved in 60 ml of water and a solution of 922.2 mg (5.98 mmol) of thiosalicylic acid in 50 ml of acetone was added. To the resulting light blue solution was added 500 mg (1.196 mmol) of artelininc acid   The mixture was stirred overnight for 18 h, during which a beige-colored precipitate appeared in the mixture.   The mixture was centrifuged (3000 rpm, 10 min) and the supernatant (pH 3) extracted with 50 ml of ethyl acetate. The extract was dried over anhydrous sodium sulphate and evaporated with a stream of nitrogen to obtain an oil.   The oil was chromatographed by preparative TLC on silica gel GF plates (20 x 20 cm; layer thickness 500 µ). Three bands were formed of which the middle band (5.5 cm - 10.6 cm) was the main one. The material in the main band was recovered and analysed by LC-MS   and then chromatographed by preparative HPLC.

2.1.1.11.   Reaction of Artelinic Acid with Iron(II) Chloride and 8-Hydroxyquinoline (HQ) in aqueous Acetone

Iron(II) chloride tetrahydrate (475.6 mg; 2.392 mmol) was dissolved in about 10 ml of water and a solution of 694.5 mg (4.78 mmol) of 8-hydroxyquinoline (oxine) in 10 ml of acetone was added. A dark blue mixture was formed immediately. Artelinic acid (200 mg; 0.479 mmol) was added and the mixture stirred overnight for 16 h. The mixture was centrifuged (3000 rpm; 15 min) and the supernatant was separated and the acetone evaporated with a stream of nitrogen. The resulting residue was extracted with ethyl acetate. The ethyl acetate extract was shaken successively with 2 x 20 ml of 0.05 M hydrochloric acid and 20 ml of distilled water and dried over anhydrous sodium sulphate.   The oily residue obtained was analysed by LC-MS and chromatographed by preparative HPLC.

2.2. RESULTS

2.2.1. Identification and Characterization of Compounds formed by the Reaction of Artelinic Acid with Complexes of Iron(II)

Extracts of reaction mixtures were initially examined by LC-MS to establish the presence of new compounds before chromatographic fractionation of the extracts was attempted. The structures of the five compounds identified after the reaction of artelinic acid with the above models systems of cytochrome P-450 are shown in Figure 3.

The total ion- and UV chromatograms of the crude product of the reaction of artelinic acid with the Udenfriend system is shown in Figure 4. These show the presence of new compounds in the extract of the reaction mixture, in addition to unchanged artelinic acid. The total ion- and UV chromatograms of the crude product of the reaction of artelinic acid with hemin and cysteine in aqueous acetone is shown in Figure 5. Identical chromatograms were obtained for the products of the reaction of artelinic acid with hemin and glutathione in aqueous acetone. Except for the absence of the peaks at 44 min and 53 min, chromatograms obtained for the products of the reaction of artelinic acid with hemin and thiosalicylic acid in acetone are also similar to those for the products of the reaction of artelinic acid with other hemin-thiol reagents. The total ion- and UV chromatograms of the products of the reaction of artelinic acid with iron(II) and thiosalicylic acid are shown in Figure 6. The peak labeled **[R]** is associated with the reagent. The chromatograms of the products of the reaction of artelinic acid with iron(II) and oxine are shown in Figure 5. The result of the reactions of artelinic acid with the iron(II) complexes are summarized in Table 1.

The identity of two other compounds (Figure 3, **[5]** and **[8]**) suspected to be formed by these reaction requires confirmation. Of the compounds identified, the previously unkown ones are the rearranged artelinic acid, 3-hydroxydeoxyartelinic acid and deoxy artelinic acid. The ^{1}H- and ^{13}C-nmr assignments for these compounds are based on the published NMR data for analogous compounds obtained by the microbial metabolism of arteether (6, 8). The spectra are also in agreement with the spectra of these compounds obtained through fungal fermentation of artelinic acid, by colleagues in the Department of Medicinal Chemistry of this institute.

The relevant nmr and mass spectra data for artelinic acid (Figure 3, ARTL, **[1]**) are provided for comparison: The ^{13}C-nmr of

artelinic acid is shown in Figure 8. The ^{1}H-nmr of artelinic acid, which is shown in Figure 9, has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton):
0.95 (3H, d, J = 5.95, Me-14),
0.98 (3H, d, J = 7.39, Me-13),
1.46 (3H, s, Me-15),
2.03 (1H, m, H-3β),
2.40 (1H, d, d, d, J = 3.90, 4.07, 3.86, H-3α),
2.72 (1H, m, H-11),
4.61 (1H, d, J = 13.32, H-16β),
4.94 (1H, d, J = 3.39, H-12),
4.99 (1H, d, J = 13.34, H-16α),
5.47 (1H, s, H-5),
7.43 (2H, d, J = 8.21, H-18, H-22),
8.10 (2H, d, J = 8.23, H-19, H-21).

The thermospray mass spectrum (m/z, relative intensity (%): 436, 3.2 %; 401, 5.2 %; 390, 11%; 373, 4.0 %; 359, 3.1 %; 355, 3.0 %; 331, 15.2 %; 284, 3.1 %; 267, 10%; 256, 28.8%; 249, 3.8 %; 221, 100 %.) is illustrated in figure 10. A fragmentation pattern which is consistent with the structure of artelinic acid is shown in Figure 11. In all the thermospray mass spectra presented, the quasi-molecular $[M + NH_4]^+$ ion is important in understanding the spectra. In some cases, this ion is quite stable and of high abundance, but sometimes it is of low abundance. The thermospray mass spectra of artelinic acid and related compounds formed in these reactions are also characterized by an apparently facile loss of the carboxybenzyl group (as carboxybenzyl alcohol, $OHCH_2PhCOOH$; 152 amu) giving rise to spectra with low abundance of the molecular or quasi-molecular ammonium adduct ions. Facile fragmentation of the carboxybenzyl side-chain also gives rise to spectra closely resembling the mass spectra of the corresponding DQHS derivatives. This is a general charateristic of the thermospray mass spectra of the ether derivatives of DQHS.

Rearranged artelinic acid (Figure 3, R-ARTL, [2]) is the major product of the reactions of artelinic acid with the iron(II) complexes. It has a melting point 186-188°. The ^{1}H-nmr, which is shown in Figure 12, has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton)

0.93 (3H, d, J = 7.51, Me-14),

0.96 (3H, d, J = 7.77, Me-13),

2.17 (3H, s, Me-15),

2.47 (1H, m, H-11),

3.95 (1H, q, J = 7.97, 8.43, 7.71, H-3β),

4.28 (1H, d, bs, d, J = 2.23, 2.10, H-3α),

4.52 (1H, d, J = 13.01, H-16β),

4.84 (1H, d, J = 4.11, H-12),

5.05 (1H, d, J = 12.91, H-16α),

6.32 (1H, s, H-5),

7.46 (2H, d, J = 8.12, H-18, H-22),

8.09 (2H, d, J = 8.17, H-19, H-21).

When compared with the spectrum of artelinic acid, the [1]H-nmr of the rearranged compound, R-ARTL, lacks the resonances at δ 2.40 and δ 2.72 associated with the two protons in the 3-position of artelinic acid. This indicates that in the transformation of artelinic acid to the rearranged compound, the 3-position of artelinic acid has been modified. Modification of the B-ring of artelinic acid (in which the 3-position is situated) and/or the C-ring is further reflected in the down-field shift in the resonance of the methyl group in the 15-position from δ 1.46 in the [1]H-nmr spectrum of artelinic acid to δ 2.17 (for a carbonyl-bonded methyl group) in the 1H-nmr spectrum of the rearranged compound, R-ARTL. The [1]H-nmr spectrum of the rearranged compound, R-ARTL, also shows the diagnostic two proton resonances at δ 3.95 and δ 4.26 which are characteristic of the alpha protons of a tetrahydrofuran ring, and which are absent in the 1H-nmr spectrum of artelinic acid.

The ^{13}C-nmr spectrum of the rearranged compound (R-ARTL) is shown in Figure 13. Comparison of the ^{13}C-nmr spectrum of R-ARTL with that of artelinic acid shows that the spectrum of R-ARTL lacks the resonance at δ 36.3 for the 3-position carbon, which corroborates the evidence from the 1H-nmr spectrum that the 3-position of artelinic acid was modified during its transformation to R-ARTL. The ^{13}C-nmr spectrum of the rearranged compound (R-ARTL) also has resonance at δ 68.5 which is absent in the ^{13}C-nmr spectrum of artelinic acid, and indicates the presence of a new oxygen-bonded

carbon atom in R-ARTL. This is agreement with the [1]H-nmr evidence for the presence of a new furan ring in R-ARTL. Further comparison of the ^{13}C-nmr spectrum of R-ARTL with that of artelinic acid shows that the spectrum of R-ARTL also lacks the resonance at δ 104.1 for the 4-position carbon, and the appearance of a new carbonyl carbon resonance at δ 169.3. This indicates that the conversion of artelinic acid to R-ARTL involved the formation of a new carbonyl function. Since the [1]H-nmr spectrum indicates that the methyl group in the 15-position is attached to the new carbonyl group, it means that it is the carbon atom in the 4-position of artelinic acid that was changed to a carbonyl carbon during the conversion of artelinic acid to R-ARTL. The [1]H-nmr and ^{13}C-nmr spectra are consistent with the structure of R-ARTL shown in Figure 3. The [1]H-nmr and ^{13}C-nmr spectra of R-ARTL are also consistent with those of the analogous rearranged artemisinin which was first reported from this institute as a product of the thermal decomposition of artemisinin, and unambiguously characterized by X-ray crystallography (14). This compound is also analogous to rearranged arteether (designated AEM1) which was found to be a microbial metabolite of arteether (9).

The thermospray mass spectrum of R-ARTL, which is illustrated in Figure 14(a), has the following peaks: (m/z, relative intensity (%): 436, 0.4 %; 401, 1.2 %; 376, 7.0 %; 359, 18.2 %; 331, 11 %; 284, 0.2 %; 242, 1.7 %; 225, 16.0 %; 207, 7.2 %; 179, 6.3 %.). The mass spectrum shows that R-ARTL has the same molecular mass as artelinic acid, thus confirming that R-ARTL was formed from the rearrangement, or isomerization of artelinic acid. Similarly, in the negative ion mass spectrum, (Figure 14(b)) the m/z 477 ion is the acetate adduct ion ($[M + CH_3COO]^-$; M+59), thus showing that the molecular mass of R-ARTL is 418 and that R-ARTL is an isomer of artelinic acid. A proposed fragmentation pattern which is consistent with the thermospray mass spectrum of R-ARTL is shown in Figure 15 Apart from features in the thermospray mass spectrum due to loss of the carboxybenzyloxy side-chain, the thermospray mass spectrum of the rearranged compound (R-ARTL) is also characterized by the M-60 peak ($[M + NH_4]^+-CH_3COOH$) resulting from the fragmentation of the acetate group.

As shown in the chromatograms of the reaction mixtures, R-ARTL was found to be a major product of the reaction of artelinic acid with all the model systems examined. The isolated yield of of this compound ranged from 4 to 6 %. with the hemin-containing systems giving yields of 15 to 20 mg of R-ARTL

3-Hydroxydeoxyartelinic acid (Figure 3, OH-deoxyARTL, [3]), was found to be another major product of the rearrangement, or isomerization of artelinic acid by the iron(II) complexes. It has a melting point of 128 - 130°. The [1]H-nmr, which is shown in Figure 16, has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton)
0.88 (3H, d, J = 6.3, Me-14),
1.00 (3H, d, J = 7.43, Me-13),
1.55 (3H, s, Me-15)
2.52 (1H, m, H-11),
3.58 (1H, q, H-3),
4.59 (1H, d, J = 13.6, H-16β),
4.86 (1H, d, J = 4.17, H-12),
4.92 (1H, d, J = 13.96, H-16α),
5.29 (1H, s, H-5),
7.41 (2H, d, J = 8.08, H-18, H-22),
8.07 (2H, d, J = 8.04, H-19, H-21).

Comparison of the [1]H-nmr spectrum of OH-deoxyARTL with that of artelinic acid shows that the [1]H-nmr spectrum of OH-deoxyARTL lacks the resonances at δ 2.40 and δ 2.72 associated with the two protons in the 3-position of artelinic acid. As with the spectrum of R-ARTL, this also indicates that in the transformation of artelinic acid to OH-deoxyARTL, the 3-position of artelinic acid has been modified. The [1]H-nmr spectrum of OH-deoxyARTL exhibits a signal (poorly resolved quartet) at δ 3.58, which indicates the presence of a methyne proton in a secondary alcohol grouping (-OHCH-) adjacent to a methylene group. This suggests that the 3-position in OH-deoxyARTL has a secondary alcohol function.
The ^{13}C-nmr of OH-deoxyARTL is shown in Figure 17. Comparison of the ^{13}C-nmr spectrum of OH-deoxyARTL with that of artelinic acid shows that the spectrum of OH-deoxyARTL lacks the resonance (δ 36.3) for the carbon atom in the 3-position, thus supporting the evidence from the [1]H-nmr spectrum that the 3-position of artelinic acid has been modified in the transformation of artelinic acid to OH-deoxyARTL. The ^{13}C-nmr spectrum of OH-deoxyARTL also exhibits the appearance of a new (and second) resonance (δ 69.5) for an oxygen-bonded carbon atom, which corroborates the evidence of the

1H-nmr spectrum for the presence of a hydroxy group on the carbon atom in position-3.

The thermospray mass spectrum of OH-deoxyARTL, which is illustrated in Figure 18(a), has the following peaks: (m/z, relative intensity (%): 436, 12 %; 401. 2 %; 284, 35 %; 266, 83 %; 249, 69 %; 231, 100 %;).

The mass spectrum shows that OH-deoxyARTL has the same molecular mass as artelinic acid, thus confirming that OH-deoxyARTL was formed from the rearrangement, or isomerization of artelinic acid. Similarly, in the negative ion mass spectrum, (Figure 18(b)) the m/z 477 ion is the acetate adduct ion ([M + CH_3COO]$^-$; M + 59), thus showing that the molecular mass of OH-deoxyARTL is 418 and that OH-deoxyARTL is an isomer of artelinic acid. A proposed fragmentation pattern which is consistent with the thermospray mass spectrum of OH-deoxyARTL is shown in Figure 19. The [1]H-nmr, ^{13}C-nmr and mass spectra are consistent with the structure of OH-deoxyARTL shown in Figure 3

3-Hydroxydeoxyartelinic acid is analogous to 3-hydroxydeoxyarteether, a known microbial and human metabolite of arteether. The [1]H-nmr and ^{13}C-nmr spectra of OH-deoxyARTL are also consistent with those of the analogous 3α-hydroxydeoxyartemisinin which was also reported from this institute as a product of the thermal decomposition of artemisinin. Isolated yields of 7 to 12 mg (percentage yield 3 to 5 %) of 3-hydroxydeoxyartelinic acid were obtained from the reactions.

Deoxyartelinic acid (Figure 3, deoxyARTL, [4]), was formed by the simple deoxygenation of artelinic acid. It had a melting point of 116-118°. The [1]H-nmr which is shown in Figure 20 has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton)
 0.89 (3H, d, J = 5.77, Me-14),
1.00 (3H, d, J = 7.40, Me-13),
 1.49 (3H, s, Me-15),
2.50 (1H, m, H-11),
4.59 (1H, d, J = 13.36, H-16β),
4.89 (1H, d, J = 4.63, H-12),
4.94 (1H, bs, H-16α),
 5.33 (1H, s, H-5),
7.43 (2H, d, J = 8.19 H-18, H-22),
8.07 (2H, d, J = 8.22, H-19, H-21).

The ^{13}C-nmr is shown in Figure 21. As might be expected the nmr spectra of deoxyARTL are very similar to that of artelinic acid. However, the thermospray mass spectrum (m/z, relative intensity (%): 420, 0.4 %; 357. 1.1 %; 268, 16.5 %; 252, 16.7 %; 251, 100 %; 233, 45.5 %; 223, 2.8 %; 215, 22.4 %; 205, 19.6 %.) shows that deoxyARTL has a molecular mass of 402, and must have been formed by the removal of one oxygen atom from artelinic acid. DeoxyARTL was found to be unresponsive to the electrochemical detector during HPLC-ECD, thus indicating that deoxyARTL must have been formed by the deoxygenation of the peroxide group of artelinic acid. The best yield of 23 mg of deoxyartelinic acid was obtained from the reaction system containing hemin and glutathione.

Analogs of DQHS, including OH-deoxyDQHS and deoxyDQHS were identified as products of the reaction of artelinic acid with the iron(II) complexes. However, these compounds are not of primary interest, since their standards are available, while the focus of the work is to obtain the unavailable, authentic standard of metabolites of artelinc acid.

3-Hydroxydeoxydihydroqinghaosu (Figure 3, OH-deoxyDQHS, [6]) formed by the reation of artelinic acid with the Udenfriend system was identified by comparison of its HPLC retention time, ^{1}H-nmr and mass spectra with those of an authentic standard. The thermospray mass spectrum (m/z, relative intensity (%): 302, 7.3 %; 285, 11 %; 284, 52.1 %; 267, 52.9 %; 266, 26.1 %; 249, 100 %; 239, 52.9 %; 231, 17.9 %; 221, 5.8 %; 207, 6.8 %.) is very similar to that of 3-hydroxydeoxyartelinic acid. As pointed out earlier, the facile fragmentation of the carboxybenzyl side-chain of artelinic acid and derivatives gives rise to thermospray mass spectra that are very similar to those of DQHS and analogs respectively. 3-OH-deoxyDQHS (2 mg) was isolated only from the reaction of artelinic acid with the Udenfriend system. In the other reactions, this compound was either not formed, or it was formed in amounts detectable only by LC-MS. In a separate work, 3-OH-deoxyDQHS was readily isolated from the reaction of DQHS itself with the Udenfriend reaction system.

Similarly, deoxydihydroqinghaosu (Figure 3,deoxyDQHS , [7]) formed by the reaction of artelinic acid with the reaction systems was also identified by comparison of its HPLC retention time and mass spectrum with those of an authentic standard. Thermospray mass spectrum (m/z, relative intensity (%): 286, 3.2 %; 268, 16.2 %; 252, 16.8 %; 251, 100 %; 233, 35.0 %; 223, 59.9 %; 215, 2.5 %; 205, 3.3 %.)

The mass spectrum of deoxyartelinic acid is almost identical to that of deoxyDQHS.

Rearranged dihydroqinghaosu (Figure 1, R-DQHS [8]) has been suggested as one of the compounds formed by the respective reaction of artelinic acid with the Udenfriend and the iron(II)-oxine systems on the basis of the similarity of its thermospray mass spectrum to that of rearranged artelinic acid. Thermospray mass spectrum of rearranged dihydroqinghaosu (m/z, relative intensity (%): 302, 4.4 %; 284, 1.6 %; 267, 2.0 %; 242, 74.7 %; 225, 19.3 %; 224, 13.1%; 207, 17.2 %; 206, 100 %; 195, 0.8 %.) This compound has not been isolated and has not been fully characterized.

LC-MS of the extract of the Udenfriend system showed that DQHS was formed in this system.

4- Hydroxymethyl benzoic acid (p-carboxybenzyl alcohol), identified by its [1]H-nmr, was recovered by TLC from the reaction of artelinic acid with the Udenfriend, iron(II)-thiosalicylic and iron(II)-oxine systems respectively.

No compound resulting from the hydroxylation of the aromatic ring of artelinic acid was observed in this study.

A possible hydroxylated artelinic acid derivative (Figure 1, [5]), with the position of the hydroxy uncertain was found to be one of the products of the reaction of artelinic acid with the hemin-based reagents and the iron(II)-8-hydroxyquinoline reagent. The amount of this compound isolated was not sufficient to allow a complete characterization of the compound. Our attempt to find the optimum reaction conditions for an enhanced yield of the hydroxylated artelinic acid derivative, and to characterize the compound form the subject of Section II of this report.

2.3. DISCUSSION

2.3.1. Study of Drug Metabolism with Chemical Models of Cytochrome P-450

The catalytic mechanism of the cytochrome P-450 enzymes has been extensively studied and chemical reactions that model biological oxygenation by the cytochrome P-450 enzymes have been an active area of interest over the last forty years. The early studies of chemical reactions that model biological oxygenation have been reviewed (15). Within the last twenty-five years however, these studies have focussed almost exclusively on the development of heme

model systems based on iron or manganese porphyrin complexes and the application of these models to the elucidation of the mechanism of dioxygen activation and oxygenation of hydrocarbons by the cytochrome P-450 enzymes.

Although many chemical models of P-450 have been developed, only very simple compounds such as cyclohexane, styrene, adamantane, aniline, toluidine, etc have been used as substrates. There have been only a few reports on the application of chemical models of the P-450 enzymes to study the metabolism of actual drugs. It was shown by Zbaida and coworkers that the reactions of Fenton reagent with cimetidine, theophylline, theobromine and caffeine simulate the hepatic metabolism of these compounds (16, 17). Similarly, Hirobe and coworkers studied the reactions of phencyclidine with a number of chemical P-450 model systems including the Udenfriend system, Fenton's reagent, and metalloporhyrin-based models (18). These workers also applied metalloporhyrin-based model systems to study the metabolic pattern of an antiasthma pyrazolo[1,5-a]pyridine derivative and to prepare an unstable, intermediate epoxide metabolite of the compound (19, 20)

Application of model systems to study drug metabolism has many attractive advantages. The most important of these in the present context are the simplicity of procedure and the formation of metabolite candidates in sufficient amounts to be used to identify the real *in vivo* metabolites. Unlike the use of model systems to study the catalytic mechanism of the cytochrome P-450 enzymes, the validating principle in the application of this concept to drug metabolism studies would be based on the identity of the products of the model reaction to the actual and expected products of hepatic metabolism, without regard to the structural similarity between the model system and the enzyme, which is a more relevant concern in mechanistic studies. Based on this consideration we examined the reaction of artelinic acid with chemical model systems of cytochrome P-450 only as means of obtaining the putative metabolites of the compound in sufficient amounts to be used for the identification of the real *in vitro* and/or *in vivo* hepatic metabolites.

2.3.2. Simulation of the Metabolism of Artelinic Acid with the Udenfriend System

The earliest of the chemical model systems of cytochrome P-450, which has come to be known as the Udenfriend system, is a non-heme system consisting of a mixture of ferrous ion, ascorbic acid, EDTA, and molecular oxygen (21). This reagent has been reported to

hydroxylate aromatic hydrocarbons as well as the saturated carbon of steroids (22). In common with the other reagents, the Udenfriend system brought about the biomimetic rearrangement of artelinic acid to the furan acetate derivative (Figure 3, R-ARTL; [2]) and 3-hydroxydeoxyartelinic acid (Figure 3, OH-deoxyARTL; [3].) This system is apparently the most effective in causing the removal of the 4-carboxybenzyl side-chain of artelinic acid thus simulating the enzymatic dealkylation of the compound. Deoxygenation of artelinic acid to deoxyartelinic acid was not observed with this reagent. Unchanged artelinic acid was also isolated from this reaction system.

There was no reaction with artelinic acid when hydrogen peroxide was added to the system, thus converting it to a Fenton reagent.

2.3.3. Simulation of the Metabolism of Artelinic Acid with the Hemin-Thiol Systems

The simulation of cytochrome P-450 by the combination of hemin and thiol-containing compounds such as cysteine and glutathione was extensively studied by Sakurai and co-workers who showed that the spectroscopic characteristics of these systems simulate those of cytochrome P-450 and that the hydroxylation of simple aromatic compounds such as chlorotoluene and toluidine by these systems is accompanied by the NIH-shift (23-28). Hydroxylation of cyclohexane to cyclohexanol by a combination of hemin and thiosalicylic acid has also been reported (29). The present work is the first attempt to apply these systems to a study of the metabolism of a drug. When the reaction of artelinic acid and the hemin-based reagents was carried out in a purely aqueous medium most of the artelinic acid was recovered unchanged, with only traces of ARTL and deoxyartelinic acid (Figure 3, deoxyARTL, [4]) being detected by LC-MS. The activity of the hemin-based reaction systems was reported to be influenced by the polarity of the medium, and it is possible that this effect is being observed here. When the reaction was carried out in aqueous acetone, a virtually complete transformation of artelinic acid was observed, with the unchanged compound being only detectable by LC-MS. In aqueous acetone,the hemin-based systems brought about the deoxygenation of artelinic acid to deoxyartelinic acid. These systems were virtually ineffective in the dealkylation of artelinic acid or the related 3-OH-deoxyartelinic acid and deoxyartelinic acid to their corresponding DQHS forms. Thus, neither DQHS nor deoxyDQHS was formed, and only traces of 3-OH-deoxyDQHS was detected, in the hemin-based systems. As stated

above, a compound suspected to be a possible hydroxyartelinic acid was also formed in the hemin-cysteine and hemin-glutathione systems. The hemin-thiosalicylic acid reagent yielded only traces of the suspected hydroxyartelinic acid.

2.3.4.   Simulation of the Metabolism of Artelinic Acid with the Iron(II)-Thiosalicylic Acid system

The combination of iron(II) with thiosalicylic acid is one of the early variations on the Udenfriend system, and this reagent was found to hydroxylate simple aromatic and aliphatic hydrocarbons (30). There has been no application of this reagent to the study of drug metabolism. No reaction was observed when the reagent was used in a purely aqueous medium. In the acetone rich medium, however, the iron(II)-thiosalicylic acid system brings about the biomimetic rearrangement, deoxygenation and dealkylation of artelinic acid. Also, a compound (Figure 4, [U]) whose mass spectrum suggests to be a possible hydroxylated artelinic acid derivative was detected by LC-MS from the iron(II)-thiosalicylic acid system. The iron(II)-thiosalicylic acid system thus simulates the putative metabolism of artelinic acid more closely than the hemin-based systems, even though the hemin-based systems may be considered structurally closer to the P-450 enzyme. The systems containing thiosalicylic acid were, however, found to be less convenient than the hemin-based systems because the poor solubility of the 2, 2'-dicarboxy diphenyldisulphide formed by the air oxidation of the thiosalicylic acid interferes in the recovery and subsequent chromatography of the desired reaction products. This, however, will not a problem in work with other artemisinin derivatives, such as arteether, which are not acids. In such cases, the 2,2'-dicarboxy diphenyldisulphide would be readily removed, after the reaction, by extraction with dilute alkali.

2.3.5.   Simulation of the Metabolism of Artelinic Acid with the Iron(II)-8-hydroxyquinoline System

The simulation of cytochrome P-450 drug metabolising activity by the combination of iron(II) and 8-hydroxyquinoline (oxine) has not been reported previously. Like the hemin-based systems, the iron(II)-oxine system caused the rearrangement of artelinic acid into R-ARTL and 3–hydroxydeoxyartelinic acid [3], its deoxygenation to deoxyartelinic acid [4] and its possible hydroxylation to a hydroxyartelinic acid. In addition, this system is capable of effecting

biomimetic dealkylation as evidenced in the formation of deoxyDQHS and the compound tentatively identified as rearranged DQHS. Thus this simple iron(II)-oxine system simulates the putative metabolism of artelinic acid more closely than the hemin-based systems.

2.4. CONCLUSION

Standards of metabolites of artelinic acid produced by the isomerization/rearrangement pathway can be produced by the reaction of the compound with simple reaction systems that simulate the cytochrome P-450 catalyzed metabolism of the compound.
This approach is simple, cheap, rapid and convenient.

3. SECTION II

3.1. BIOMIMETIC HYDROXYLATION OF ARTELINIC ACID

3.1.1. Introduction

As shown in the work described above, our attempts to chemically simulate the metabolism of artelinic acid by reaction of artelinic acid with different complexes of iron(II) gave rise mostly to products formed by the isomerization and deoxygenation pathways (Figure 1). While the work described above was in progress, a number of reports appeared on the reaction of artemisinin and other 1,2,4-trioxanes with ferrous salts, in studies aimed at elucidating the mechanism of antimalarial action of these compounds (31, 32, 33, 34)

All the reports showed that iron(II) salts caused the isomerization of artemisinin to the furano acetate derivative and 3-hydroxydeoxyartemisinin, in a manner analogous to our own observation on the isomerization of artelinic acid to the corresponding furano acetate isomer and 3-hydroxydeoxyartelinic acid by complexes of iron(II).

The challenge then was to use the iron(II) complexes to catalyse the hydroxylation of artelinic acid while avoiding its isomerization. The reports on the isomerization of artemisinin by iron(II) salts only encouraged our own approach of using the iron(II) complexes rather than the iron(II) salts. It was thought that the complexed iron(II) ion might be less oxophilic than the non-complexed, relatively 'naked' ion in the salt form. Thus the iron complex might be less reactive towards the peroxide group of artelinic acid and, therefore, less able to cause the isomerization

and/or deoxygenation of artelinic acid, while at the same time retaining enough reactivity towards dioxygen to be able to catalyze the hydroxylation of artelinic acid. In other words, we speculated that the complex of iron(II) should be able to mimic the action of cytochrome P-450 on artelinic acid more closely than the inorganic salts of iron(II), especially with regards to the hydroxylation of artelinic acid.

The direct chemical hydroxylation of saturated (or unactivated) hydrocarbons (C-hydroxylation) usually requires very drastic conditions of high temperature, high pressure and the use of strongly electrophilic reagents. Such drastic C-hydroxylation methods cannot be applied to artelinic because such conditions would only cause the degradation of the artelinic acid molecule. In contrast to chemical reagents, C-hydroxylation of an unactivated carbon-hydrogen bond takes place readily, and under mild conditions, in biological systems by use of enzyme systems such as the iron-porphyrin based cytochrome P-450 or the non-heme iron-protein ω-hydoxylase. To the chemist, therefore, simulation of these enzyme systems remains both a practical and intellectually interesting challenge.

Hydrocarbon hydroxylation may be viewed as the insertion of an oxygen atom into the C-H bond to give a C-OH group., i.e. as an oxygenation:

$$\begin{array}{c} | \\ -C-H \\ | \end{array} \xrightarrow{[O]} \begin{array}{c} | \\ -C-OH \\ | \end{array}$$
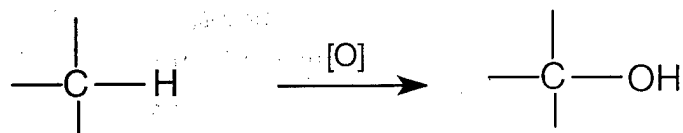
Enzyme systems capable of oxygenating hydrocarbons are known to do so because of their ability to activate dioxygen. Non-enzymic reactions which may bring about the activation of dioxygen would, therefore, be expected to cause the oxygenation of the C-H bond under mild conditions. Such reactions would be considered to be 'biomimetic oxygenation reactions'. The desire to hydroxylate artelinic acid under mild conditions led us to a study of reactions of metal complexes that might bring about the activation of dioxygen.

There is a kinetic barrier to oxidations by dioxygen which results from two properties of dioxygen. Namely:

i. Ground state dioxygen is a triplet state

ii. Lowest orbital available to accept an electron is a partially-filled anti-bonding π-orbital, making a one-electron reduction of dioxygen difficult.

Processes that help to overcome these kinetic barriers to oxidation are said to cause the activation of dioxygen. Activation of dioxygen may involve excitation, reduction or coordination.

Excitation is the perturbation of the π^*-orbitals of dioxygen without an electron transfer:

$$\pi^* \quad \uparrow \quad \uparrow \xrightarrow{\text{23 kcal/mol}} \quad \uparrow\downarrow$$
$$\quad\quad {}^{3}\Sigma_g \quad\quad\quad\quad\quad\quad {}^{1}\Delta_g$$
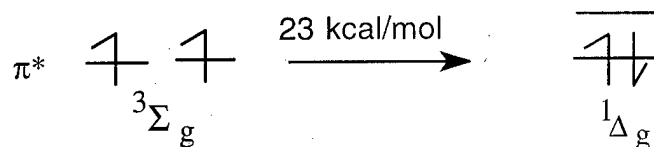
Succesive one-electron reduction of dioxygen produces superoxide ion, hydrogen peroxide and a hydroxy radical

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-,\ H^+} H_2O_2 \longrightarrow HO\cdot + HO^-$$
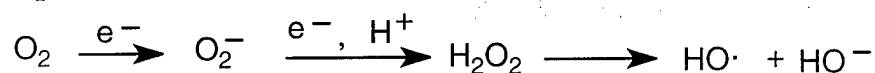
Ground state dioxygen may be activated through coordination to transition metal complexes.   The iron-porhyrin complex of cytochrome P-450 is an example of such a complex.   The analogic reasoning which suggests other complexes of iron(II) as possible mimics of the iron-porphyrin complex of cytochrome P-450 is quite obvious.   The metal accomplishes the activation of dioxygen essentially by bringing about the addition of an electron into the antibonding π-orbital of dioxygen through strong donation of π-electrons into the π^*-orbitals.   The triplet character of ground-state dioxygen is also eliminated as indicated by the diamagnetic nature of the metal-oxygen complexes.

Addition of dioxygen to many ferrous chelates results in oxidation of the iron, not an iron-oxygen complex. In the oxidation of the metal, electrons must be transferred from the iron to the dioxygen, followed by the separation of a negatively charged dioxygen species and a more positively charged iron. Thus the oxidation would be inhibited by a medium of low dielectric constant.

Furthermore, it should be noted that the same iron-porphyrin complex contained in hemoglobin, cytochromes in the electron-transfer system, tryptophan 2,3-dioxygenase and cytochrome P-450 displays different behaviour depending on the environment where the complex is located. That the consequences of the interaction of these complexes with oxygen would be sensitive to the environment

of the complex, is also shown clearly by the difference in the behaviour of the iron-porphyrin complex of hemoglobin towards oxygen, and the behaviour of the same complex in cytochrome P-450. Based on these observations, we, therefore, studied the reaction of artelinic acid with the various complexes of iron(II) and manganese(II) in various solvents.

Studies with synthetic porphyrins have shown that iron-porphyrin complexes formed from porphyrins which are highly substituted with electronegative groups, such as halogens and the nitro group, are more reactive in the hydroxylation of hydrocarbons that the iron complexes of unsubstituted porphyrins. By analogy, therefore, we studied the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinolines which are substituted with electronegative groups in the 5-position to see if these would be more effective than 8-hydroxyquinoline itself in bringing about the hydroxylation of artelinic acid.

The attempt to hydroxylate artelinic acid using the complexes of iron(II) as catalysts requires that the complex and the conditions of the reaction be such that would bring about C-hydroxylation of artelinic acid while avoiding the much more facile isomerization pathway. With the thinking that one way to inhibit isomerization is to reduce the oxophilicity of the complex, and assuming the presence of secondary ligands might reduce the oxophilicity of the complex, we studied the reactions of artelinic acid with the complexes of iron(II) and manganese(II) in presence of mercaptoethanol and/or pyridine as possible secondary ligands.

Studies with synthetic porphyrins have shown that synthetic manganese-porphyrin complexes bring about oxygen activation in the same way as synthetic iron-porphyrin complexes. Both classes of complexes have been used to catalyse olefin epoxidation (35, 36). Many non-porphyrinic complexes of manganese(II) have also been shown to be sensitive to dioxygen, clearly suggesting that they may act as oxygenation catalysts (37). Guided by these observations, we also studied the reaction of artelinic acid with complexes of manganese(II).

3.2. MATERIALS AND METHODS
Chemicals

All reagents and chemicals were obtained from Aldrich Chemical Co (Milwakee, USA) except artelinic acid and sodium artelinate which were from the Walter Reed Inventory.

Instrumentation

3.2.1. Isolation of Reaction Products

Preparative thin layer chromatography was carried out using 20cm x 20cm tappered layer plates (Uniplate, Analtech Inc, Newark, DE, USA) with a hexane:acetone:acetonitrile (75/25/5, v/v/v) mixture as mobile phase.

Preparative HPLC was performed on a Waters liquid chromatography system consisting of two Waters model 510 solvent delivery units, a U6K injector and a Waters model 440 UV detector set at 254 nm. A Waters u Bondapak C_{18} preparative column (7.8 mm x 300 mm; 10 mm) was used with a mobile phase consisting of 0.1M ammonium acetate buffer (pH 4.5) and acetonitrile. Elution was done with a stepwise gradient starting with 80:20 (v/v) 0.1M ammonium acetate:acetonitrile and maintained for 70 min and changed to a 70:30 ratio (70 min to 92 min), then changed to a 60:40 ratio (from 92 min to 110 min). and finally maintained at a 50:50 ratio (from 92 to 140 min). Mobile phase flow rate was 2.0 ml/min.

For HPLC, the reaction products were dissolved in methanol just before injection. It was found that keeping a solution of the reaction products in methanol for two or more hours resulted in a very significant decrease in the peak size of one of the compounds which is identified as OH-ARTL below. In situations where all the crude reaction product could not be chromatographed at once, the material was dissolved in dichloromethane and portions of this solution were evaporated and the residue redissolved in methanol just before injection into the HPLC.

After each run, the column was washed with pure acetonitrile for 20 min follwing the injection of pure methanol to clean the injection port. Acetonitrile was removed from the collected fractions with a stream of nitrogen and the fractions were then extracted with ethyl acetate (3 ml) by shaking on a vortex mixer for 2 min. The ethyl acetate extracts were dried with anhydrous sodium sulphate and evaporated with a stream of nitrogen. The residues were crystallised from a mixture of diethyl ether and hexane.

For the qualitative examination of the reaction products, a slightly shorter gradient was also used, consisting of the following steps: 80:20 (v/v) 0.1M ammonium acetate:acetonitrile (from 0 to 60 min); 70:30 ratio (60 min to 90 min), then 50:50 ratio (from 90 to 120 min). Mobile phase flow rate was 2.0 ml/min.

3.2.2. Characterization of Reaction Products

Mass spectrometric identification of the reaction products was performed using a HPLC-MS system consisting of a Hewlett Packard 1090 Liquid Chromatograph System linked with a Hewlett Packard HP 5989A Mass Spectrometer via a Hewlett Packard thermospray interface. A μBondapak C_{18} column (2.1 mm x 100 mm; 5 μ) was used, with a mobile phase consisting of 0.1M ammonium acetate (pH 4.5) and acetonitrile. Elution was done with either of two linear gradients: 95:5 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 30:70 ratio at 60 min, and held at this ratio for a further 20 min (flow rate 0.4 ml/min), or 90:10 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 60:40 ratio at 60 min, and held at this ratio for a further 30 min (flow rate 0.4 ml/min). The thermospray interface was operated in the "fragmenter on" mode at a vaporiser temperature of 85-96°C (or 87-105°C) and a source temperature of 220°C.

Negative ion mass spectrometry was carried out using on a HPLC-MS system consisting of a Hewlett Packard 1050 Liquid Chromatograph System linked with Finnegan LCQ Mass Spectrometer. A μBondapak C_{18} column (4.6 mm x 150 mm; 5 μ) was used, with a mobile phase consisting of 25mM ammonium acetate (pH 4.5) and acetonitrile. Elution was done with a linear gradient of 90:10 (v/v) 25mM ammonium acetate:acetonitrile maintained for 10 min and then increasing to a 60:40 ratio until 60 min, and held at this ratio for a further 20 min (flow rate 1.0 ml/min). Ionization was by Atmospheric Pressure Chemical Ionization (APCI), with the discharge needle set at 5kV.

[1]H- and ^{13}C-nmr spectra were obtained on a Brucker AC 300 Spectrometer, using $CDCl_3$ as solvent and Me_4Si as internal standard.

Melting points (uncorrected) were determined on a Thomas Hoover Unimelt[R] capillary melting point apparatus.

3.2.3. Reaction of artelinic acid with iron(II) chloride and 8-hydroxyquinoline (HQ) in aqueous acetone

Iron(II) chloride tetrahydrate (119 mg: 0.6 mmol) was dissolved in about 10 ml of water and a solution of 174 mg (1.2 mmol) of 8-hydroxyquinoline in 10 ml of acetone was added. A dark blue mixture was formed immediately. Artelinic acid (50 mg; 0.12 mmol) was added and the mixture stirred overnight for 18 h. The mixture was centrifuged (3000 rpm; 15 min) and the supernatant was separated and the acetone removed evaporated with a stream of

nitrogen. The resulting residue was extracted with ethyl acetate. The ethyl acetate extract was shaken successively with 2 x 20 ml of 0.5 M hydrochloric acid and 20 ml of distilled water and then dried over anhydrous sodium sulphate. The oily residue obtained was analysed by LC-MS and chromatographed by gradient HPLC.

3.2.4. Reaction of artelinic acid with iron(II) sulfate and 8-hydroxyquinoline (HQ) in aqueous acetone

A mixture of Iron(II) sulphate ($FeSO_4.7H_2O$; 166mg; 0.6mmol) and 5ml of water was sonicated in an ultrasonic bath for 15min before adding to a solution of 174mg (1.2mmol) of hydroxyquinoline in 25ml of acetone. More water (2.5ml) and acetone (5ml) were added to the dark blue mixture, followed by 50mg (0.12mmol) artelinic acid. The mixture was stirred at room temperature for 18h and the centrifuged at 3000rpm for 15min. The supernatant was removed and the acetone evaporated with a stream of nitrogen. Water (10ml) was added, and the aqueous mixture was then extracted with 30ml of ethyl acetate by shaking on a vortex mixer for 3min. The ethyl acetate extract was washed twice with 15ml of dilute hydrochloric acid. The ethyl acetate extract was dried over anhydrous sodium sulfate and the solvent vaporated with a stream of nitrogen. The oily residue obtained was examined by gradient HPLC and by LC-MS.

3.2.5. Reaction of artelinic acid with iron(II) chloride and 8-hydroxyquinoline (HQ) in a mixture of acetone and mercaptoethanol

Sixty milligrams (0.3mmol) of iron(II) chloride were dissolved in 25ml of acetone in a 250ml round-bottom flask by stirring on a magnetic stirrer for 5min. Hydroxyquinoline (87mg; 0.6mmol) was added, and the mixture stirred at room temperature for 15min. Mercaptoethaol (1.5ml) was then added, followed by 50mg (0.12mmol) of artelinic acid. The mixture was stirred at room temperature for 21h and the transferred to a 50-ml plastic test tube. The solvent was evaporated with a stream of nitrogen, and the residue reconstituted in 30ml of ethyl acetate. The ethyl acetate solution was washed with thrice with 20ml of dilute hydrochloric acid, and twice with 20ml of distilled water.
The ethyl acetate solution was dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. The oily residue obtained was examined by gradient HPLC and by LC-MS.

3.2.6. Reaction of artelinic acid with iron(II) chloride and 8-hydroxyquinoline (HQ) in a mixture of acetone and pyridine

Sixty millihrams (0.3mmol) of iron(II) chloride was dissolved in 25ml of acetone in a 250ml round-bottom flask by stirring on a magnetic stirrer for 5min. Hydroxyquinoline (87mg; 0.6mmol) was added, and the mixture stirred at room temperature for 15min. Pyridine (1.5ml) was then added, followed by 25 mg (0.12mmol) of artelinic acid. The mixture was stirred at room temperature for 21h and then transferred to a 50-ml plastic test tube. The solvent was evaporated with a stream of nitrogen, and the residue reconstituted in 30ml of ethyl acetate. The ethyl acetate solution was washed with thrice with 20ml of dilute hydrochloric acid, and twice with 20ml of distilled water.

The ethyl acetate solution was dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. The oily residue obtained was examined by gradient HPLC and by LC-MS.

3.2.7. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of acetone and mercaptoethanol

8-Hydroxy-5-nitroquinoline (114mg; 0.6 mmol) was dissolved in 5 ml of acetone in a 250ml round-bottom flask and 60mg (0.3 mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The mixture was stirred with a magnetic stirrer at room temperature for 30min. Mercaptoethanol (2 ml) was added to the mixture, followed by 50 mg (0.12 mmol) of artelinic acid. The mixture was stirred at room temperature for 20h.

The acetone was evaporated with a stream of nitrogen and the dark residue was redissolved mixed with 25ml of ethyl acetate and the ethyl acetate solution transferred to a 50-ml plastic test tube. The ethyl acetate solution was washed repeatedly with dilute hydrochloric acid (2M) by shaking on a vortex mixer until it turned light yellow. The ethyl acetate solution was then dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. The oily residue obtained was examined by gradient HPLC and by LC-MS.

3.2.8. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of acetone and pyridine

8-Hydroxy-5-nitroquinoline (114mg; 0.6mmol) was placed in a 250ml round-bottom flask and mixed with 12ml of acetone and 60mg (0.3mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The dark green

mixture was stirred with a magnetic stirrer at room temperature for 30min. Pyridine (6ml) was added to the mixture, followed by 50mg (0.12mmol) of artelinic acid. The mixture was stirred at room temperature for 22h.

The solvent was evaporated with a stream of nitrogen. The dark residue was redissolved in about 15ml of ethyl acetate and the ethyl acetate solution transferred to a 50-ml plastic test tube. The ethyl acetate solution was washed repeatedly with dilute hydrochloric acid (2M) by shaking on a vortex mixer until it turned light yellow. The ethyl acetate solution was then dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. The oily residue obtained was examined by gradient HPLC and by LC-MS.

3.2.9. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in pure acetone

The preceeding experiment was repeated, but using 30 ml of pure acetone as solvent.

3.2.10. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in pure pyridine

The experiment described in section 3.2.8 was repeated, but using 10 ml of pure pyridine as solvent.

3.2.11. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in dichloromethane, in presence of imidazole

8-Hydroxy-5-nitroquinoline (57mg; 0.3mmol) was placed in a 250ml round-bottom flask and mixed with 20ml of dichloromethane and 30mg (3mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The mixture was stirred at room temperature for 1h. Imidazole (100mg; 1.5mmol) was then added, followed, after a futher 15min of stirring, by the addition of 25mg (0.06mmol) of artelinic acid. The reaction mixture was stirred at room temperature for 18h.

The reaction mixture was washed with dilute hydrochloric acid (2M) as described above. The oil obtained upon evaporation of the dichloromethane also was examined by TLC, gradient HPLC and by LC-MS.

3.2.12. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in pure dichloromethane

The procedure described in section 3.2.8 was repeated, but using 30 ml of dichloromethane as solvent.

3.2.13. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in pure benzene

The procedure described in section 3.2.8 was repeated, but using 25 ml of pure benzene as solvent.

3.2.14. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in pure ethanol

The procedure described in section 3.2.8 was repeated, but using 25 ml of absolute n ethanol as solvent.

3.2.15. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of pyridine and ethanol

8-Hydroxy-5-nitroquinoline (57mg; 0.3mmol) was placed in a 250ml round-bottom flask and mixed with 3ml of absolute ethanol and 30mg (3mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The dark green mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (7ml) was added to the mixture, followed by 25mg (0.06mmol) of artelinic acid. The mixture was stirred at room temperature for 14h.

The solvent was evaporated with a stream of nitrogen. The dark residue was redissolved in about 50ml of dichloromethane and the dichloromethane solution washed with 45ml of dilute hydrochloric acid (2M) by stirring with the magnetic stirrer for about 5min. The aqueous layer was removed with a Pasteur pipet. The acid washing was repeated until the dark dichloromethane solution turned pale yellow, signifying the destruction of the iron(II) complex of NHQ.

The dichloromethane solution was dried over anhydrous sodium sulfate and solvent evaporated with a stream of nitrogen. An oil was obtained, which was examined by TLC, gradient HPLC and by LC-MS

The procedure was repeated using solvents consisting of other different proportions of methanol and pyridine.

3.2.16. Reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of pyridine and methanol

The preceeding experiment was repeated, but using methanol in place of methanol. The reaction was also performed in solvents consisting of other different proportions of methanol and pyridine.

3.2.17. Reaction of artelinic acid with iron(II) and 8-hydroxyquinoline (HQ) in a mixture of pyridine and ethanol

8-Hydroxyquinoline (44 mg; 0.3 mmol) was dissolved in 22.5 ml of absolute ethanol, in a 250 ml round-bottom flask, iron(II) chloride was aded, and the mixture stirred at room temperature for 30 min, Pyridine (2.5) ml was added, followed by 25 mg of artelinic acid, and the mixture stirred for 22h. The solvent was evaporated with a stream of nitrogen. The dark residue was reconstituted in 20 ml of ethyl acetate, and the solution stirred vigorously with about 30 ml of dilute hydrochloric acid on a magnetic stirrer for 5 min. The mixture was transferred to a 50 ml test tube, and centrifuged at 3000rpm for 15 min. The ethyl acetate layer was removed and washed again with 20 ml dilute hydrochloric acid by shaking on a vortex mixer for 3 mix. The water-white ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated with a stream of nitrogen. The clear oil obtained, was analysed by gradient HPLC.

3.2.18. Reaction of artelinic acid with iron(II) and 5-chloro-8-hydroxyquinoline (CHQ) in a mixture of pyridine and methanol

5-Chloro-8-hydroxyquinoline (66mg; 0.3mmol) was placed in a 250ml round-bottom flask and mixed with 3ml of absolute methanol and 30mg (3mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The dark blu-black mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (7ml) was added to the mixture, followed by 25mg (0.06mmol) of artelinic acid. The mixture was stirred at room temperature for 20h.

The solvent was evaporated with a stream of nitrogen. The dark residue was reconstituted in 30ml of ethyl acetate and the mixture was stirred vigorously with 30ml of dilute hydrochloric acid (2M) for about 5min. The mixture was centrifuged (3500rpm for 15min) and the ethyl acetate layer removed. The ethyl acetate extract was washed with another portion of dilute hydrochloric acid, upon which it turned water-white. The ethyl acetate extract was dried over anhydrous sodium sulfate and then evaporated with a stream of nitrogen. The resulting residue was examined by gradient HPLC.

3.2.19. Reaction of artelinic acid with iron(II) and 5, 7-dichloro-2-methyl-8-quinolinol (DCMHQ) in a mixture of pyridine and methanol

5, 7-Dichloro-2-methyl-8-quinolinol (137mg; 0.6mmol) was placed in a 250ml round-bottom flask and mixed with 3ml of

absolute methanol and 30mg (3mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The dark blue mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (7ml) was added to the mixture, followed by 50mg (0.12mmol) of artelinic acid. The mixture was stirred at room temperature for 3h.

The solvent was evaporated with a stream of nitrogen. The dark residue was reconstituted in 30ml of ethyl acetate and the mixture washed repeatedly with dilute hydrochloric acid, as described above, until the ethyl acetate layer became light yellow in color. After drying the ethyl acetate extract over anhydrous sodium sulfate and then evaporating the solvent with a stream of nitrogen, a yellow residue obtained and was examined by gradient HPLC.

3.2.20. Reaction of artelinic acid with iron(II) and 5, 7-dibromo-2-methyl-8-quinolinol (DBMHQ) in a mixture of pyridine and methanol

The preceding experiment was repeated, except that 196mg (0.6mmol) of 5, 7-dibromo-2-methyl-8-quinolinol was used in place

3.2.21. Reaction of artelinic acid with iron(II) and 8-hydroxyquinoline-5-sulfonic acid (SHQ) in a mixture of pyridine and methanol

8-Hydroxyquinoline-5-sulfonic acid (SHQ) (135mg; 0.6mmol) was placed in a 250ml round-bottom flask and mixed with 3ml of absolute methanol and 60mg (6mmol) of iron(II) chloride ($FeCl_2.4H_2O$). The leaf-green mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (7ml) was added to the mixture, upon which it turned dark blue. Addition of pyridine was immediately followed by the addition of 50mg (0.12mmol) of artelinic acid. The mixture was stirred at room temperature for 3h. The solvent was evaporated with a stream of nitrogen. The dark residue was redissolved in about 35ml of dichloromethane and the dichloromethane solution washed with about 50ml of dilute hydrochloric acid (2M) by stirring with the magnetic stirrer for about 5min. A clear dichloromethane layer was obtained

The dichloromethane solution was dried over anhydrous sodium sulfate and solvent evaporated with a stream of nitrogen. A clear oily was obtained, which was examined by TLC, gradient HPLC and by LC-MS

3.2.22. Reaction of artelinic acid with iron(II) and 8-hydroxyquinoline-5-sulfonic (SHQ) in a mixture of pyridine and ethanol

The preceeding experiment was repeated, but using absolute ethanol in place of methanol.

3.2.23. Semi-preparative reaction of artelinic acid with iron(II) and 8-hydroxyquinoline-5-sulfonic acid (SHQ) in a mixture of pyridine and methanol

8-Hydroxyquinoline-5-sulfonic acid (SHQ) (1350mg; 6mmol) was placed in a 250ml round-bottom flask and mixed with 10ml of absolute methanol and 600mg (6mmol) of iron(II) chloride ($FeCl_2.4H_2O$). The mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (24ml) was added to the mixture, immediately followed by the addition of 500mg (1.2mmol) of artelinic acid. The mixture was stirred at room temperature for 4h. The mixture was divided into 5 portions in plastic 50-ml test tubes and the solvent was evaporated with a stream of nitrogen. The dark residue from each portion was redissolved in about 30ml of ethyl acetate and the ethyl acetate solution repeatedly washed with dilute hydrochloric acid (2M) by shaking on a vortex mixer for 2 mins. The clear ethyl acetate extracts were pooled and dried over anhydrous sodium sulfate. The solvent was evaporated with a stream of nitrogen to give an off-white solid.

For preparative TLC, the solid was redissolved in dichloromethane and applied to 5 tappered-layer silica gel plates. The plates were developed in a mixture of hexane/acetone/acetonitrile (72/25/5, v/v/v). Four bands were obtained. To recover the material in the bands, the silica gel was scraped into test tubes and extracted with an ethyl acetate/methanol (80/20) mixture by shaking on a vortex mixer for 2min. The mixture was centrifuged (3500 rpm for 15min) and separated solvent evaporated with a stream of nitrogen. The fastest-moving narrow band (rf = 0.56) contained unreacted artelinic acid. This is followed by the largest band in the chromatogram (rf = 0.47), which was shown to contain rearranged artelinic acid. The third band (rf = 0.34) was shown to contain 3-hydroxydeoxyartelinic acid. The fourth band (band 4) had a rf of 0.25. The solid obtained from slowest moving band (band 4) was shown by LC-MS to be a mixture of a monohydroxylated- and a dihyroxylated artelinic acid derivative. This material was, therefore, further chromatographed by gradient HPLC to separate this two compounds.

Alternatively, TLC of the crude reaction product was done on silica gel using a mixture of benzene/methanol/acetic acid (95/5/5, v/v/v) as mobile phase. A chromatogram consisting of 6 bands was obtained. The fastest-moving and also the largest band (band1) was shown to contain rearranged artelinic acid. The second band was narrow and probably contained material derived from the reagent. The third band was shown to contain hydroxydeoxyartelinic acid, while the 4th and 5th bands contained monohydroxylated- and dihydroxylated artelinic acid derivatives respectively. The slowest-moving 6th band is made up of poorly resolved colored bands containing reagent-derived material. The compounds contained in bands 1, 3, 4 and 5 were extracted from the silica gel as described above, using ethyl acetate/methanol (80/20) mixture. After evaporating the solvent with a stream of nitrogen, the residues were recrystallized from a mixture of diethyl ether and hexane.

3.2.24. Semi-preparative reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of pyridine and methanol

8-Hydroxy-5-nitroquinoline (570mg; 3mmol) was placed in a 250ml round-bottom flask and mixed with 3ml of absolute methanol and 300mg (3mmol) of iron(II) chloride (FeCl$_2$.4H$_2$O) . The dark green mixture was stirred with a magnetic stirrer at room temperature for 1h. Pyridine (7ml) was added to the mixture, followed by 250mg (0.6mmol) of artelinic acid. The mixture was stirred at room temperature for 14h.

The solvent was evaporated with a stream of nitrogen. The dark residue was redissolved in about 50ml of dichloromethane and the dichloromethane solution washed with 45ml of dilute hydrochloric acid (2M) by stirring with the magnetic stirrer for about 5min. The aqueous layer was removed with a Pasteur pipet. The acid washing was repeated until the dark dichloromethane solution turned pale yellow, signifying the destruction of the iron(II) complex of NHQ.

The dichloromethane solution was dried over anhydrous sodium sulfate and solvent evaporated with a stream of nitrogen to obtain a yellow oily residue. Preparative TLC and HPLC were carried out as described in the preceeding experiment.

3.2.25. Semi-preparative reaction of artelinic acid with iron(II) and 8-hydroxyquinoline-5-sulfonic acid (SHQ) in dichloromethane, in presence of imidazole

8-Hydroxyquinoline-5-sulfonic acid (675mg; 3mmol) was placed in a 250ml round-bottom flask and stirred with 50ml of dichloromethane

for 15min. SHQ was found to be insoluble in dichloromethane and on addition of 300mg (30mmol) of iron(II) chloride ($FeCl_2.4H_2O$) no apparent reaction was observed after stirring the mixture for 1h at room temperature. On adding methanol (10ml) and imidazole (410mg; 6mmol) a dark blue mixture was formed. Artelinic acid (250mg; 0.6mmol) was then added, and the reaction mixture was stirred at room temperature for 5h. The solvent was evaporated with a stream of nitrogen, because it was necessary to remove the methanol. The residue was then reconstituted in dichloromethane. Dilute hydrochloric acid (100ml) was added, and the mixture stirred on the magnetic stirrer for about 10min. The mixture was transferred to a separating funnel and the aqueous layer remove. The dichloromethane layer was returned to the round-bottom flask and stirred with a fresh portion of dilute hydrochloric acid. The acid washing procedure was repeated until the dicholorimethane layer turned to a light yellow color. The dichloromethane solution was then dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. A clear oil was obtained.

Preparative thin layer chromatography of the oil was carried out on tappered silica gel plates with a hexane:acetone:acetonitrile (75/25/5, v/v/v) mixture as solvent. The three separated bands were scraped into test tubes and the silica gel extracted with an ethyl acetate:methanol (80/20,v/v) mixture by shaking on a vortex mixer for 2 to 3min. After centrifuging, the solvent was removed and evaporated with stream of nitrogen. The residues obtained were recrystallized from a mixture of ether and hexane.

In this reaction, no dihydroxylated artelinic acid is formed. Thus after TLC of the crude product the material extracted from the fastest moving band (rf = 0.41) was shown to be pure rearranged artelinic acid, while the second band (rf = 0.28) contained hydroxydeoxyartelinic acid, and the slowest moving band (rf = 0.19) contained the monohydroxylated artelinic acid derivative

3.2.26. Semi-preparative reaction of artelinic acid with iron(II) and 8-hydroxy-5-nitroquinoline (NHQ) in dichloromethane, in presence of imidazole

8-Hydroxy-5-nitroquinoline (570mg; 3mmol) was placed in a 250ml round-bottom flask and stirred with 50ml of dichloromethane for 15min., followed by the addition of 300mg (30mmol) of iron(II) chloride ($FeCl_2.4H_2O$) . The dark green mixture was stirred at room temperature for 1h, and imidazole (410mg; 6mmol) was then added, followed, after a futher 15min of stirring, by the addition of 250mg

(0.6mmol) of artelinic acid. The reaction mixture was stirred at room temperature for 3h.

Dilute hydrochloric acid (100ml) was added, and the mixture stirred on the magnetic stirrer for about 10min. The mixture was transferred to a separating funnel and the aqueous layer remove. The dichloromethane layer was returned to the round-bottom flask and stirred with a fresh portion of dilute hydrochloric acid. The acid washing procedure was repeated until the dicholorimethane layer turned to a light yellow color. The dichloromethane solution was then dried over anhydrous sodium sulfate, and the solvent evaporated with a stream of nitrogen. An orange-colored oil was obtained. Preparative thin layer chromatography of the oil was carried out on tappered silica gel plates with a hexane:acetone:acetonitrile (75/25/5, v/v/v) mixture as solvent. The three separated bands were scraped into test tubes and the silica gel extracted with an ethyl acetate:methanol (80/20,v/v) mixture by shaking on a vortex mixer for 2 to 3min. After centrifuging, the solvent was removed and evaporated with stream of nitrogen. The first two bands contained rearranged artelinic acid and hydroxydeoxyartelinic acid respectively, with rearranged artelinic acid being the faster moving compound. The material recovered from the slowest moving band (third band) was repeatedly rechromatographed on 150 μ silica gel plates to remove a yellow pigment that was present in the material. The purified material exhibited a mass spectrum which suggests it is a hydroxylated artelinic acid derivative.

3.2.27. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex

3.2.27.1. Preparation of manganese(II)-8-hydroxyquinoline complex

In a 250ml round-bottom flask, 8-hydroxyquinoline (3g; 0.021mol) was dissolved, with warming, in 80ml of absolute ethanol. To the warm solution was added 4.5g (0.023mol) of manganese chloride ($MnCl_2.4H_2O$). A golden-yellow solution forms immediately, from which a a yellow precipitate soon appears. The mixture was stirred at room temperature for 24 h. The mixture was filtered by suction, and the filtrate evaporated to give a dark-yellow solid which mixed with acetone and then filtered to give a yellow powder.

3.2.27.2. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex in a mixture of acetone and mercaptoethanol

Artelinic acid (50mg; 0.12mmol) was in 3ml of acetone in a 100ml round-bottom flask, The manganese-8-hydroxyquinoline complex (50mg) was added, followed by 2ml of mercaptoethanol. The mixture was stirred at room temperature for 14h. The solvent was evaporated with a stream of nitrogen, the residue reconstituted in 30ml of ethyl acetate, and the ethyl acetate solution washed thrice with 15ml of dilute hydrochloric acid. The ethyl acetate solution was dried over anhydrous sodium sulfate and the evaporated with a stream of nitrogen. The oily residue obtained was analysed by gradient HPLC.

3.2.27.3. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex in pure pyridine

Artelinic acid (25mg; 0.06mmol) and 50mg of the manganese-8 hydroxyquinoline complex were dissolved in 3ml of pyridine, and the mixture stirred at room temperature for 18h. The solvent was evaporated and the residue reconstituted in ethyl acetate, and the ethyl acetate treated as described in the preceeding section. The oily residue obtained was analysed also by gradient HPLC.

3.3. RESULTS

3.3.1. Investigation of the optimum conditions for the formation of hydroxylated artelinic acid derivatives by the reaction of artelinic acid with iron(II) and 8-hydroxyquinolines

In Section I of this report it was pointed out that a compound whose mass spectrum suggested to be a hydroxylated artelinic acid derivative, was one of the products of the reaction of artelinic acid with the iron(II)-8-hydroxyquinoline complex. The aim of Section II of this work was to determine the optimum reaction conditions for the formation of the hydroxylated artelinic acid derivative, so that enough of the compound could be isolated to permit its characterization, and thereby establishing a simple reaction for the biomimetic hydroxylation of artelinic acid.

The results of the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline and its substituted analogs, in different solvents, are summarized in Table 2.

The chromatogram obtained following gradient HPLC of the products of the reaction of artelinic acid with the complex of iron(II) and 8-hydroxyquinoline in aqueous acetone is shown in Figure 22. The peaks labeled R-ARTL and OH-deoxyARTL are due to rearranged artelinic acid and hydroxydeoxyartelinic acid respectively. The characterization of these two compounds have been fully described in Section I of this report. The peak labeled OH-R-ARTL is due to a possible hydroxylated artelinic acid derivative. In these investigations the aim was to increase the yield of the hydroxylated artelinic acid derivative relative to the yield of R-ARTL and OH-deoxyARTL. In other words, reaction conditions were being sought which would allow the biomimetic hydroxylation of artelinic acid by the iron(II) complexes to predominate over the isomerization pathway that leads to R-ARTL and OH-deoxyARTL. The relative yield of the hydroxylated artelinic acid derivative under the different reaction conditions was judged by comparison of the OH-R-ARTL peak to the OH-deoxyARTL peak.

The relatively small size of the OH-R-ARTL peak in figure 21 shows that the yield of the hydroxylated artelinic acid derivative was small when artelinic acid was reacted with iron(II) chloride and 8-hydroxyquinoline in a medium of aqueous acetone.

The first change in the reaction condition that was examined was to use iron(II) sulfate in place of iron(II) chloride. A HPLC chromatogram of the reaction products is shown in Figure 23, which shows a dramatic difference from the result shown in Figure 22. Figure 23 shows that rearranged artelinic acid was the sole product when artelinic acid was reacted with iron(II) sulfate and 8-hydroxyquinoline. (The other small peaks in the chromatogram are reagent related.)

The dramatic difference in the results obtained when iron(II) chloride or iron(II) sulfate was used suggested that secondary ligands would have a significant effect on the course of the reaction of artelinic acid with the complex of iron(II) and 8-hydroxyquinoline. The reaction was, therefore carried out in presence of mercaptoethanol or pyridine, two solvents that could conceivably coordinate with the iron(II)-hydroxyquinoline complex. A HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxyquinoline in a mixture of mercaptoethanol and acetone is shown in Figure 24. This shows the absence of the peak for the hydroxylated artelinic acid derivative OH-R-ARTL. Figure 23 also shows there is no unchanged artelinic acid and that rearranged artelinic acid is the predominant product. A HPLC chromatogram of the products of the reaction of artelinic acid

with iron(II) chloride and 8-hydroxyquinoline in a mixture of acetone and pyridine is shown in Figure 25. Although this chromatogram suggest the presence of a trace of OH-R-ARTL, most of the artelinic acid (ARTL) remained unchanged when the reaction was carried out in presence of pyridine.

The above results suggested that improvement in the yield of the hydroxylated artelinic acid derivative, OH-R-ARTL, would not be achieved by changing the reaction solvent alone. It was decided, therefore, to investigate the effect of using 8-hydroxyquinolines substituted with electronegative groups in place of 8-hydroxyquinoline itself. The rationale for this investigation has been discussed in the introduction to Section II. The structures of the substituted 8-hydroxyquinolines investigated are shown in Figure 26

The first substituted 8-hydroxyquinoline to be investigated was 8-hydroxy-5-nitroquinoline (NHQ). A HPLC chromatogram of the products of the reaction of artelinic acid with NHQ in a mixture of acetone and mercaptoethanol is shown in Figure 27. This chromatogram is similar to that of Figure 24 and shows the absence of the peak for OH-R-ARTL.

A HPLC chromatogram of the products of the reaction of artelinic acid with NHQ in a mixture of acetone and pyridine is shown in Figure 28. A comparison of the chromatogram in Figure 28 with that in Figure 25 shows a remarkable increase in the relative yield of the OH-R-ARTL derivative when 8-hydroxy-5-nitroquinoline was used in place of 8-hydroxyquinoline in a reaction medium containing pyridine. A comparison of the chromatogram of Figure 28 with that of Figure 25 also shows that 8-hydroxy-5-nitroquinoline is more reactive in this reaction than the unsubstituted 8-hydroxyquinoline since there is relatively less of the unchanged artelinic acid when 8-hydroxy-5-nitroquinoline was used in place of 8-hydroxyquinoline. A comparison of Figures 27 and 28 again suggests that the presence of mercaptoethanol inhibits the formation of the hydroxylated artelinic acid derivative, OH-R-ARTL, (and promotes the isomerization of artelinic acid to R-ARTL and OH-deoxyARTL) while the presence of pyridine enhances the formation of OH-R-ARTL.

To confirm the beneficial effect of pyridine in promoting the formation of the hydroxylated artelinic acid derivative (OH-R-ARTL) the reaction of iron(II) chloride and NHQ with artelinic acid was carried out in pure acetone. A chromatogram of the reaction products is shown in Figure 29. This shows the absence of the peak due to OH-R-ARTL as well as the fact that rearranged artelinic acid (R-ARTL) is the only substantial product, although most of the

artelinic acid remained unreacted. In contrast, as illustrated in the chromatogram in Figure 30, when the reaction was carried out in pure pyridine, artelinic acid was completely transformed to the hydroxylated derivative (OH-R-ARTL) and the isomerization products (R-ARTL and OH-deoxyARTL).

To determine whether the ability of pyridine to promote the reaction of artelinic acid with the iron(II)-NHQ complex is related to its ability to act as a secondary ligand to the complex, the reaction of artelinic acid with iron (II) and NHQ was carried out in dichloromethane in presence of imidazole. It is expected that imidazole could coordinate with the iron(II)-NHQ complex, through the nitrogen atom, the same way pyridine could. A chromatogram of the reaction products obtained when artelinic acid was reacted with iron(II) chloride and NHQ in dichloromethane and in the presence of imidazole is shown in Figure 31. This chromatogram is virtually identical to the chromatogram in Figure 30, showing that imidazole influences the reaction in the same way as pyridine. The effect of imidazole on the reaction was further confirmed by reacting artelinic acid with iron(II) chloride and NHQ in pure dichloromethane. A chromatogram of the reaction product is illustrated in Figure 32 which shows that rearranged artelinic acid (R-ARTL) is virtually the sole product, with no OH-R-ARTL being formed.

Comparison of the result when the reaction was carried out in pure dichloromethane (Figure 32) to the result when the reaction was carried out in pure acetone (Figure 29) suggests that the iron(II)-NHQ complex is less reactive in acetone, thereby leaving much of the artelinic acid unchanged. In contrast, there was no unchanged artelinic acid when the reaction was carried out in pure dichloromethane. In contrast with the results obtained when the reaction was carried out in pure dichloromethane, when the reaction was carried out in solvent mixtures containing different proportions of pyridine and dichloromethane, the chromatograms obtained were identical to that obtained when the reaction was carried out in pure pyridine (Figure 30.) This, again, confirms the role of pyridine in promoting the formation of the hydroxylated artelinic acid derivative, OH-R-ARTL. A solvent composition of 7% pyridine/93% dichloromethane was found to be optimum for the formation of OH-R-ARTL.

Since both acetone and dichloromethane may be considered as non-coordinating solvents, the difference in the reactivity of the iron(II)-NHQ complex in acetone and dichloromethane was thought to be related to differences in the polarity of the two solvents. The effect of solvent polarity on the extent and course of the reaction of

artelinic acid with the iron(II)-NHQ complex was, therefore, investigated. The reaction was carried out in pure benzene, a non-polar and non-coordinating solvent. A chromatogram of the reaction product is illustrated in Figure 33 which shows that artelinic acid was completely converted to the isomerization products (R-ARTL and OH-deoxyARTL) and the hydroxylated artelinic acid derivative (OH-R-ARTL) was not formed. When the reaction was carried out in a mixture of 30% pyridine/70% benzene, a chromatogram identical to that in Figure 33 was obtained thus suggesting that the action of pyridine in promoting the formation of OH-R-ARTL was suppressed by the opposing effect of the relatively low polarity of the medium.

To further investigate the effect of the polarity of the medium on the extent and course of the reaction of artelinic acid with the iron(II)-NHQ complex, the reaction was carried out in absolute ethanol. A chromatogram of the reaction products is shown in Figure 34. This shows that some of the artelinic acid was converted to the isomerization products (R-ARTL and OH-deoxyARTL) and the hydroxylated artelinic acid derivative (OH-R-ARTL) was not formed.

The reaction of artelinic acid with the iron(II)-NHQ complex was then carried out in a mixture of ethanol and pyridine. A chromatogram of the reaction products is illustrated in Figure 35. This shows that both the isomerization products (R-ARTL and OH-deoxyARTL) and the hydroxylated artelinic acid derivative (OH-R-ARTL) were formed. In addition, a new major product was formed whose mass spectrum showed to be a dihydroxyartelinic acid derivative (di-OH-ARTL). The reaction of artelinic acid with the iron(II)-NHQ was then studied in solvents containing different proportions of pyridine and ethanol. A solvent composition of 70% pyridine/30% ethanol was found to be optimun for the formation of the hydroxylated artelinic acid derivatives (OH-R-ARTL and di-OH-ARTL.). As described above, semi-preparative reaction of artelinic acid was later carried out in this solvent mixture for the purpose of isolation and characterization of the hydroxylated artelinic acid derivatives. Methanol behaved similarly as ethanol.

Having previously established, as described above, that a substituted 8-hydroxyquinoline, such as NHQ, was more effective in this reaction than 8-hydroxyquinoline itself, the reactivity of the iron(II) complex of other substituted 8-hydroxquinolines other than NHQ was investigated. First, the reactivity of the iron(II)-8-hydroxyquinoline complex in a mixture of pyridine and ethanol was studied by reacting artelinic acid with iron(II) chloride and 8-hydroxyquinoline in a mixture of pyridine and ethanol. A chromatogram of the reaction products obtained is illustrated in

Figure 36. This shows that one of the isomerization products (R-ARTL) was the major product of the reaction, and that much unreacted artelinic acid remained. This is in sharp contrast to the result illustrated in Figure 35, and confirms the relatively higher reactivity of 8-hydroxy-5-nitroquinoline (NHQ) as determined in other solvents.

Next, the reactivity of other 8-hydroxyquinolines substituted with electron-withdrawing groups other than the nitro group was investigated to see if their iron(II) complexes would be more effective than 8-hydroxyquinoline itself in converting artelinic acid to the hydroxylated derivatives, OH-ARTL and di-OH-ARTL. After reacting artelinic acid with iron(II) chloride and 5-chloro-8-hydroxyquinoline (CHQ) in a mixture of methanol and pyridine, the chromatogram obtained is identical to that illustrated in Figure 36, showing that as in the reaction with 8-hydroxyquinoline itself, most of the artelinic acid remained unchanged while a little of the rearranged compound (R-ARTL) was formed. Thus, 5-chloro-8-hydroxyquinoline is not any more reactive than 8-hydroxyquinoline in this reaction.

Artelinic acid was reacted with iron(II) chloride and 5,7-dichloro-2-methyl-8-quinolinol in a mixture of methanol and pyridine. A chromatogram of the reaction products is illustrated in Figure 37, which shows evidence of the formation of the hydroxylated artelinic acid derivatives (OH-R-ARTL and di-OH-ARTL) as well as the isomerization products (R-ARTL and OH-deoxyARTL). This is similar to the result obtained with 8-hydroxy-5-nitroquinoline (NHQ) (Figure 35). (The large peak at the later part of the chromatogram in Figure 37 is reagent related.) Artelinic acid was reacted with iron(II) chloride and 5,7-dibromo-2-methyl-8-quinolinol in a mixture of methanol and pyridine. A chromatogram of the reaction products is illustrated in Figure 38, which also shows evidence of the formation of the hydroxylated artelinic acid derivatives (OH-R-ARTL and di-OH-ARTL) as well as the isomerization products (R-ARTL and OH-deoxyARTL).

Artelinic acid was reacted with iron(II) chloride and 8-hydroxyquinoline-5-sulfonic acid (SHQ) in a mixture of methanol and pyridine. A chromatogram of of the reaction products is illustrated in Figure 39 . This shows a relatively high yield of the hydroxylated artelinic acid derivatives (OH-R-ARTL and di-OH-ARTL) compared to the results obtained with other substituted 8-hydroxyquinolines.

It should be noted that the primary aim of this work was to achieve the biomimetic hydroxylation of artelinic acid no attempt

was made to determine the exact nature of the iron complexes of the 8-hydroxyquinolines involved in the reactions.

3.3.2. Isolation and characterization of the hydroxylated artelinic acid derivatives produced by the reaction of artelinic acid with iron(II) chloride and substituted 8-hydroxyquinolines

Since the reaction of artelinic acid with NHQ or SHQ in a mixture of pyridine and ethanol or in the presence of imidazole seem to give the best yields of the hydroxylated artelinic acid derivatives (OH-R-ARTL and di-OH-ARTL) the semi-preparative reaction of artelinic with NHQ or SHQ was carried out as described, to isolate and characterize the hydroxylated artelinic acid derivatives.

The monohydroxylated compound, (OH-R-ARTL, figure 43) has a melting point of 214-216°. The ^{1}H-nmr (Figure 40) has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton):
δ 0.90 (3H, d, J = 6.35, Me-14),
0.98 (3H, d, J = 7.28, Me-13),
1.89 (1H, m, H-11),
2.19 (3H, s, Me-15),
4.53 (1H, d, J = 12.91, H-16β),
4.83 (1H, d, J = 4.1, H-12),
5.05 (1H, d, J = 12.87, H-16α),
6.28 (1H, unresolved triplet, H-3),
6.35 (1H, s, H-5),
7.46 (2H, d, J = 8.20, H-18, H-22),
8.08 (2H, d, J = 8.26, H-19, H-21).

Comparison of the ^{1}H-nmr of OH-R-ARTL with that of artelinic acid shows that the resonance for the methyl group in the 15-position which appears as a 3-proton singlet at $\delta 1.46$ in artelinic acid is absent in the ^{1}H-nmr of the OH-R-ARTL. Instead, the 3-proton singlet appears at δ 2.19, which indicates a methyl group adjacent to a carbonyl function. The δ value of 2.19 for the 15-methyl group of OH-R-ARTL is comparable to the δ value of 2.17, for the same methyl group in rearranged artelinic acid as described above (Section I). The resonance for the single proton in the 5-position of OH-R-ARTL has a δ value of 6.35, the same as in the spectrum of rearranged artelinic acid. This suggests that the unknown OH-R-ARTL is a

hydroxylated rearranged artelinic acid. The [1]H-nmr of OH-R-ARTL is, however, devoid of the diagnostic two proton resonances at δ 3.95 and δ 4.26 characteristic of the α protons of a tetrahydrofuran ring as found in rearranged artelinic acid (R-ARTL). This indicates that the hydroxy group of OH-R-ARTL is present in the 3-position, that is, the α-position of the tetrahydrofuran ring of the rearranged compound. Signals at δ 2.03 and δ 2.40 for the respective 3α and 3β protons of artelinic acid are absent from the spectrum of OH-R-ARTL, thus confirming that the 3-position has been altered in OH-R-ARTL. Hydroxylation of the α-position of the tetrahydrofuran ring of the rearranged compound introduces a new -OCHO- grouping, the presence of which proton is indeed confirmed by the signal at δ 6.28. As would be expected, this is comparable to the δ value of 6.35 for the proton in the 5-position which is in another, similar -OCHO- grouping. The [1]H-nmr spectrum is consistent with the structure of OH-R-ARTL depicted in Figure 43 as hydroxylated rearranged artelinic acid. The stereochemistry of the hydroxy group is not certain.

The thermospray mass spectrum of OH-R-ARTL is illustrated in Figure 41(a): (m/z, relative abundance (%): 452, 100 %; 434, 24 %; 417, 9 %; 392, 14 %; 374, 31 %; 357, 12 %; 300, 23 %; 283, 5 %; 256, 18 %; 240, 30 %; 223, 15 %; 205, 28 %. The negative ion mass spectrum is also illustrated in figure 40(b): (m/z, relative abundance (%): 493, 70%; 433, 100%; 373, 24 %; 337, 30 %; 223, 25 %.
The highest mass in the thermospray mass spectrum (m/z 452) is due to the quasi-molecular [M + NH4]$^{+}$ ion. This means that the molecular mass of OH-R-ARTL is 434, which is 16 units above the molecular mass of artelinic acid, thus showing that OH-R-ARTL was formed by the addition of an oxygen atom to the artelinic acid molecule. A fragmentation patern consistent with the thermospray mass spectrum is illustrated in Figure 42. As in the mass spectrum of the related rearranged artelinic acid (Figures 14 and 15), M-60 (that is, [M - CH3COOH]) ions formed by the loss of the acetate group are a prominent feature of the thermospray mass spectrum of OH-ARTL.

The negative-ion mass spectrum of OH-R-ARTL (Figure 41(b)) exhibits a quasi-molecular [M + CH3COO]$^{-}$ adduct ion with m/z of 493 (that is, M + 59). The peak at m/z 433 is due to the [M - H]$^{-}$ ion. These features of the negative-ion mass spectrum again show that the molecular mass of OH-R-ARTL is 434 and that it is a monohydroxylated artelinic acid derivative. The structure of OH-R-

ARTL depicted in Figure 43 is also consistent with our observation mentioned earlier that the compound apparently decomposes when a solution of the compound in a polar solvent such as methanol is kept for some time. HPLC of a methanol solution of the compound show, within hours, a decrease in the size of the peak due to OH-R-ARTL and the appearance of new peaks. The decomposition of OH-R-ARTL may be accounted for as being due to the opening of the hemi-acetal ring of the structure depicted in Figure 43. A solution of the compound in a less polar solvent, such as dichloromethane, was found to be relatively more stable. The yield of OH-R-ARTL in the reactions was between 5 and 7 %

The dihydroxylated compound (Figure 43, di-OH-ARTL) has a melting point of 120-122°. The ^{1}H-nmr (Figure 44) has the following resonances:

δ (number of protons, multiplicity of signal, J-value, position of proton):
δ 0.98 (3H, d, J = 6.72, Me-13),
 1.21 (3H, d, J = 7.10, Me-14),
 1.45 (3H; singlet, Me-15)
 2.10 (2-H, d, d, d, J = 2.72, 2.99, 2.65; H-2),
 2.63 (1-H, m, H-11)
3.65 (1-H, q, H-9)
3.81 (1-H, t, H-3)
4.53 (1H, d, J = 12.91, H-16β),
4.79 (1H, d, J = 4.1, H-12),
4.90 (1H, d, J = 12.87, H-16α),
5.20 (1H, s, H-5),
7.41 (2H, d, J = 8.29, H-18, H-22),
8.10 (2H, d, J = 8.28, H-19, H-21).

The resonances at δ 3.65 and 3.81,which are absent in the spectrum of artelinic acid, indicate that the compound has two new secondary alcohol functions, that is, it has two new -HOCH- groupings. The four possible positions for the newly introduced secondary hydroxy groups are the 2, 3, 8 and 9 positions. When compared with the ^{1}H-nmr of artelinic acid, the spectrum of di-OH-ARTL lacks the resonance signals for the two position-3 protons at δ 2.03 and 2.40. This suggests that one of the hydroxy groups of di-OH-ARTL is in the 3-position. This is corroborated by the appearance of the resonance of the protons in position-2 as three doublets centered at δ 2.10. The triplet signal centered at δ 3.81 was, therefore, assigned to the proton

in the 3-position of di-OH-ARTL. The second hydroxy group of di-OH-ARTL could be either in position-8 or position-9. The significnant shift in the resonance of methyl group in the 14-position (from δ 0.95 in the ^{1}H-nmr spectrum of arteinic acid to δ 1.21 in the spectrum of di-OH-ARTL) suggests that the second hydroxy group is close this methyl group. that is, it is in the 9-position. Based on the ^{13}C-nmr evidence. as discussed below, the second hydroxy group of di-OH-ARTL is thought to be at the position-9. Thus, in the ^{1}H--nmr of di-OH-ARTL, the quartet of signals centered at δ 3.65 is due to the proton in the 9-position.

The ^{13}C-nmr of the compound is illustrated in Figure 45. When compared with the ^{13}C-nmr spectrum of artelinic acid, the spectrum of di-OH-ARTL shows two new signals at δ 72.1 and d 73.0 which indicate that two new oxygen-bonded carbons atoms are present in the molecule of di-OH-ARTL. In the ^{13}C-nmr spectrum of artelinic acid, the carbon atom in the 3-position resonates at δ 36.287. This signal in absent in the ^{13}C-nmr spectrum of di-OH-ARTL, thus providing further evidence that one of the hydroxy groups of di-OH-ARTL is in the 3-position. Similarly, in the ^{13}C-nmr spectrum of artelinic acid, the resonance signal for the carbon atom in the 9-position occurs at δ 34.464. This signal is absent in the spectrum of di-OH-ARTL, thus indicating that the other hydroxy group of di-OH-ARTL was introduced into the 9-position. This is also consistent with the shift in the resonance of the carbon atom in the 8-position from δ24.412 in the ^{13}C-nmr spectrum of artelinic acid to δ 31.93 in the spectrum of di-OH-ARTL. This shift arises because the oxygenated 9-position in di-OH-ARTL is adjacent to the carbon atom in the 8-position. For the same reason, a similar shift is also observed in the resonance of the carbon atom in the 10-position, from δ 37.309 in artelinic acid to δ 49.200 in di-OH-ARTL

The thermospray- and negative ion mass spectra of di-OH-ARTL are illustrated in Figures 46(a) and 46(b). The highest mass in the thermospray mass spectrum (m/z 468) is due to the quasi-molecular [M + NH4]$^+$ ion. This means that the molecular mass of di-OH-ARTL is 450, which is 32 units above the molecular mass of artelinic acid, thus showing that di-OH-ARTL was formed by the addition of two oxygen atoms to the artelinic acid molecule. A fragmentation pattern proposed for the thermospray mass spectrum is illustrated in Figure 47. The presence of a M-32 (M-CH3OH) peak in the thermospray spectrum of di-OH-ARTL is consistent with the

presence of hydroxy group which is adjacent to a methyl group as in the proposed structure of di-OH-ARTL. In the negative ion mass spectrum, the m/z 509 ion is the acetate adduct ion ($[M + CH_3COO]^-$; $M + 59$), thus showing that the molecular mass of di-OH-ARTL is 450 and that di-OH-ARTL must have been formed from artelinic acid by the addition of two oxygen atoms. Thus, all the spectroscopic evidence suggest that the sructure of di-OH-ARTL is as depicted in Figure 43. The stereochemistry of the hydroxy groups is not certain.

The yield of di-OH-ARTL in the reaction was between 8 and 10 %

3.3.3. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex in a mixture of acetone and mercaptoethanol

Artelinic acid reacted completely with the manganese(II)-8-hydroxyquinoline complex, in an acetone-mercaptoethanol mixture, to give almost exclusively the rearranged compound (R-ARTL), with only a trace of OH-deoxyARTL.

3.3.4. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex in pure pyridine

Artelinic acid remained virtually unchanged when it was reacted with the manganese(II)-8-hydroxquinoline complex in pure pyridine, with only a trace of the rearranged compound (R-ARTL) being formed.

3.4. DISCUSSION

3.4.1. Biomimetic hydroxylation of artelinic acid with complexes of 8-hydroxyquinoline and its analogues

There has been no previous report on C-hydroxylation using the iron(II) complexes of 8-hydroxyquinolines. Therefore, our attempt to hydroxylate artelinic acid using these complexes required a thorough study of the reaction conditions. In these reactions, the hydroxylation pathway is in competition with the pathway that leads to the isomerization, or rearrangement, of artelinic acid. As described above, the effectiveness of the reaction system in bringing about the hydroxylation of artelinic, under any particular reaction

condition, could easily be judged by comparing the relative yields of the rearrangement and hydroxylation products.

The rationale for our choice of reaction conditions, or factors, to be studied have been detailed in the introductory part to Section II of this report. The three main factors studied are the polarity of the solvent, the effect of possible secondary ligands, and the structure of the 8-hydroxyquinoline analogs.

The above results show that the reaction of artelinic acid with the complexes of 8-hydroxyquinoline analogs is inhibited in polar solvent such as acetone or ethanol. In these solvents, much of the artelinic acid remained unreacted, and only one of the isomerization products is the sole, or major product. In contrast, in a relatively non-polar solvent such as benzene, artelinic acid was completely comverted to a mixture of the isomerization products, R-ARTL and OH-deoxyARTL. However, the hydroxylation of artelinic acid was not observed in this solvent.

The effect of possible secondary ligands was studied by carrying out the reaction in the presence of pyridine or imidazole or mercaptoethanol. Pyridine and imidazole could coordinate with the iron(II)-8-hydroxyquinoline complex through their respective nitrogen atoms, while mercaptoethanol could coordinate through both an oxygen and a sulfur atom. The reactivity of the iron(II)-8-hydroxyquinoline complex would, therefore, be expected to be affected in different ways by pyridine and imidazole on the one hand, and mercatoethanol on the other. The results described above show that mercaptoethanol promotes the isomerization of artelinic acid by the iron(II)-8-hydroxyquinoline complex. For example, while the reaction is inhibited when pure acetone was used as solvent, artelinic acid was completely converted to a mixture of the isomerization products when the acetone contained a little of mercaptoethanol. However, mercaptoethanol did not promote the hydroxylation of artelinic acid. Pyridine was also found to promote the reaction of the iron(II)-8-hydroxyquinoline complex with artelinic acid. When the reaction was carried out in a mixture of acetone and pyridine, both the isomerization products and the monohydroxylated artelinic acid derivative were formed, although some unreacted artelinic acid remained, reflecting the inhibiting effect of acetone. This result show that pyridine promotes the hydroxylation of artelinic acid by the iron(II)-8-hydroxyquinoline complex. When the reaction was carried out in pure pyridine, artelinic acid was completely converted to the isomerization products and the monohydroxylated rearranged artelinic acid derivative (OH-R-ARTL). Since the polarity index of pyridine (5.3) is similar to that

of acetone (5.1), it is unlikely that the different, respective effects of the two solvents on the reaction is due to differences in their polarity. The behavior of pyridine in promoting the hydroxylation of artelinic acid by the iron(II)-8-hydroxyquinoline complex is more likely to be due to the ability of pyridine to coordinate with the complex through its nitrogen atom. This reasoning is suported by the result of the reaction of artelinic acid with the complex of iron(II) and the substituted 8-hydroxyquinolines in the presence of imidazole. When the reaction was carried out in dichloromethane in which imidazole was dissolved, the result obtained was identical to that obtained when the reaction was carried out in pure pyridine. Since imidazole is not a solvent, its effect on the reaction could only have arisen from its ability to coordinate with the complex through a nitrogen atom, in the same manner as pyridine. Thus the influence of the solvent in this reaction may derive not from the polarity effect, but from the efffect of possible coordination of solvent molecules with the iron(II)-8-hydroxyquinolines complex. This is borne out by the result obtained when the reaction was carried out in a mixture of pyridine and methanol or a mixture of pyridine and ethanol. When the reaction was carried out in a mixture of pyridne and either of these alcohols both monohydroxylated and dihydroxylated artelinic acid derivatives were formed, in addition to the products of isomerization. As noted above, the reaction of artelinic acid with the iron(II) complexes of the 8-hydroxyquinolines occurs only to a limited extent in absolute ethanol or methanol. Yet, when used as a mixture with pyridine these alcohols apparently augment the effect of pyridine in the reaction, to the extent that dihydroxylation of artelinic acid was acheived Since the polarity index of the alcohols (5.1) is the same as that of pyridine the influence of the alcohols in enhancing the hydroxylation of artelinic by the iron(II)-8-hydroxyquinoline complex is probably related to their effect on the coordination of pyridine to the complex, rather than a polarity effect. It is, however, possible that the high dielectric constant of the alcohols, as well as the higher solubility of dioxygen in the alcohols play a role in their apparent ability to augment the hydroxylation of artelinic acid by the iron(II)-8hydroxyquinoline complex in presence of pyridine.

It was noted in the introduction to Section II that activation of dioxygen by iron(II) complexes involves oxidation of the metal. It is also well known that certain complexes of iron(II), such as the iron-salen complex, interact so strongly with atmospheric dioxygen that the complexes can only be isolated under an inert atmosphere. Interaction of such complexes with dioxygen results in the oxidation

of the iron(II) to iron(III). Since this is an electron transfer process, it would be expected that structural features of the ligand which may influence the electron transfer process would affect the activation of dioxygen by the complex, and hence the ability of the complex to bring about C-hydroxylation. It was with this thinking in mind that we studied the reaction of artelinic acid with complexes formed from iron(II) and different 8-hyroxyquinolines which have electron-withdrawing groups in the 5-position of the 8-hydroxyquinoline molecule. The results reported above show that the iron(II) complex of 8-hydroxyquinoline itself did not bring about the hydroxylation of artelinic acid when the reaction was carried out in a mixture of pyridine and methanol or ethanol. In contrast, 8-hydroxy-5-nitroquinoline (NHQ) and 8-hydroxyquinoline-5-sulfonic which respectively have the electron-withdrawing nitro- and sulfonic groups in the 5-position, for iron(II) complexes which react with artelinic acid in a mixture of ethanol (or methanol) and pyridine to bring about the mono- and dihydroxylation of the artelinic acid. The effect of electron-withrawing groups in the benzene ring of the 8-hydroxyquinoline in promoting hydroxylation by the iron(II) complex is demonstrated by the contrasting results obtained with the respective iron(II) complexes of 5-chloro-8-hydroxyquinoline (CHQ) and 5, 7-dichloro-2-methyl-8-quinolinol (DCMHQ). As with the iron(II) complex of the unsubstituted 8-hydroxyquinoline, no hydroxylation was observed in the reaction of the iron(II) complex of CHQ with artelinic acid in an ethanol-pyridine mixture. In contrast, with DCMHQ which has a second chlorine substituent in the benzene ring of the quinoline, the iron(II) complex brings about both mono- and dihyroxylation of artelinic acid.

In summary, the present results show that hydroxylation of artelinic acid (and perhaps other artemisinin derivatives) may be achieved by reaction with the iron(II) complexes of 8-hydroxquinolines which have strongly electron-withdrawing groups in the 5-position of the quinoline ring, with the reaction being carried out in the presence of pyridine or imidazole.

3.4.2. Reaction of artelinic acid with manganese(II)-8-hydroxyquinoline complex

The reaction of artemisinin compounds with the manganese(II) complex of 8-hydroxyquinoline has not been previously reported. Artelinic acid was reacted with this complex to find out if this could bring about the hydroxylation of artelinic acid. The results obtained show that the manganese(II) complex of 8-hydroxyquinoline only brings about the isomerization of isomerization of artelinic, with

almost exclusive formation of the furan acetate derivative, R-ARTL. The results also show that the reaction of the manganese(II)-8-hydroxyquinoline complex with artelinic acid is promoted by mercaptoethanol, while it is inhibited by pyridine. Since the substitution of manganese(II) for iron(II) in these reactions did not seem to have any apparent advantage the reaction of artelinic acid with the manganese(II) complex of 8-hydroxyquinolines was not studied as extensively as the reaction with the iron(II) complexes.

3.4.3. Correlation of the biomimetic reaction with the *in vitro* metabolism of artelinic acid by rat liver microsomes

As noted earlier in this report, the reaction of artelinic acid with the iron(II) complex of the 8-ahydroxyquinolines brings about the isomerization of artelinic acid to give the furan acetate derivative (R-ARTL) and 3-hydroxydeoxyartelinic (OH-deoxyARTL) in the same way as observed with the reaction of artelinic acid with rat liver microsomes. Thus, the biomimetic reaction offers a ready means of producing standards of the isomerization metabolites.

A monohydroxylated artelinic acid has been observed as a microsomal metabolite of artelinic acid. A chromatogram of the compounds formed on the incubation of artelinic acid with rat liver microsomes in shown in Figure 48, by courtesy of Dr Skanchy. Comparison of this chromatogram with the chromatograms presented in Section II of this work shows that the hydroxylated artelinic acid metabolite elutes just before OH-deoxyARTL as observed with the OH-R-ARTL identified from the biomimetic reaction. However, similarity of chromatographic behaviour is no proof of identity. Like the microsomal metabolite, the compound OH-R-ARTL produced in this work also respond to the electrochemical detector during HPLC with electrochemical detection, which may be taken to mean that the peroxide group of artelinic acid remains intact in both OH-R-ARTL and the microsomal metabolite. However, the compound OH-R-ARTL, as the hydroxyaldehyde isomer, may respond to the EC detector because the aldehyde group is reducible at the voltage at which the detector is used to detect the artemisinin compounds. There is therefore no certainty that the hydroxylated rearranged artelinic acid (OH-R-ARTL) produced by the biomimetic reaction is identical to the hydroxyartelinic acid produced by the microsomal metabolism of artelinic acid.

Unlike the biomimetic reaction, metabolic dihydroxylation of artelinic acid has not been observed. However, dihydroxylation of

artelinic acid as reported here may serve as a means of functionalizing the 3- and 9-positions of the molecule.

4.   SUMMARY AND CONCLUSIONS

1.   The essential problem confronted in the study of the metabolism of artelinic acid, as in the study of the metabolism of other artemisinin derivatives, is the non-availability of authentic standards of putative metabolites to use in the unambiguous identification of real *in vitro* and *in vivo* metabolites.

2.   In the present work we attempted to overcome this problem by studying the reaction of artelinic acid with complexes of iron(II) and manganese(II), which are chemical systems that might simulate the action of the cytochrome P-450 enzymes.

3.   Biomimetic isomerization of artelinic acid to the furan acetate isomer and 3-hydroxydeoxyartelinic acid, and its deoxygenation to deoxyartelinic acid, were readily effected by its reaction with complexes of iron(II) and manganese(II) in an appropriate solvent.

4.   The isomerization of artelinic acid by these complexes is promoted by mercaptoethanol, but inhibited by pyridine.

5.   Biomimetic mono- and dihydroxylation of artelinic acid was achieved by the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline analogs substituted in the 5-position with electron-attracting groups, and in the presence of pyridine or imidazole.

6.   Monohydroxylation alone was achieved when the reaction was carried out in pure pyridine or in dichloromethane containing imidazole.

7.   Both mono- and dihydroxylation were achieved when the reaction was carried out in mixture of pyridine and ethanol (or methanol)

8.   The monohydroxylated compound underwent rearrangement to the corresponding furan acetate derivative.

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TABLE 1

Products of the reaction of artelinic acid with iron(II) complexes

REACTION SYSTEM	PRODUCTS					
	Unchanged ARTL	R-ARTL	OH-deoxyARTL	deoxyARTL	OH-deoxyDQHS	Possible hydroxylated ARTL
Udenfriend system	x	X	X	X	x	
Hemin-Cysteine in water	X	−	−	−	−	−
Hemin-Cysteine in aqueous acetone	−	X	X	x	x	x
Hemin-Glutathione in water	X	−	−	−	−	−
Hemin-Glutathione in aqueous acetone	−	X	X	x	x	x
Hemin-Thiosalicylic acid in water	X	trace	trace	−	−	−
Hemin-Thiosalicylic acid in acetone	trace	X	X	x	x	x
Iron(II)-Thiosalicylic acid in water	X	−	−	−	−	−
Iron(II)-Thiosalicylic acid in acetone	trace	X	X	x	x	
Iron(II)-8-hydroxyquinoline in aqueous acetone	trace	X	X	x	x	x

Note: x = minor product
X = major product

Table 2
Products of the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline and its substituted analogs.

REACTION SYSTEM	PRODUCTS				
	Unchanged ARTL	R-ARTL	OH-deoxyARTL	OH-R-ARTL	di-OH-ARTL
Iron(II) + HQ in aqueous acetone	–	X	X	x	–
Iron(II) sulfate + HQ in aqueous acetone	–	X	–	–	–
Iron(II) + HQ in acetone + mercaptoeth-anol	–	X	X	–	–
Iron(II) + HQ in acetone + pyridine	X	x	x	–	–
Iron(II) + NHQ in acetone + mercaptoeth-anol	–	X	x	–	–
Iron(II) + NHQ in acetone + pyridine	X	X	X	X	–
Iron(II) + NHQ in pure acetone	X	x	–	–	–
Iron(II) + NHQ in pure pyridine	–	X	X	X	–

Table 2 (contd.)
Products of the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline and its substituted analogs.

REACTION SYSTEM	PRODUCTS				
	Unchanged ARTL	R-ARTL	OH-deoxyARTL	OH-R-ARTL	di-OH-ARTL
Iron(II) + NHQ + imidazole in dichlorome-thane	—	X	X	X	—
Iron(II) + NHQ in pure dichlorome-thane	—	X	trace	—	—
Iron(II) + NHQ in pure benzene	—	X	X	—	—
Iron(II) + NHQ in pure ethanol	X	X	trace	—	—
Iron(II) + NHQ in pyridine + ethanol	X	X	X	X	X
Iron(II) + NHQ in pyridine + methanol	X	X	X	X	X
Iron(II) + HQ in pyridine + ethanol	X	x	trace	—	—
Iron(II) + CHQ in pyridine + ethanol	X	x	trace	—	—

Table 2 (contd.)
Products of the reaction of artelinic acid with the iron(II) complexes of 8-hydroxyquinoline and its substituted analogs.

REACTION SYSTEM	PRODUCTS				
	Unchanged ARTL	R-ARTL	OH-deoxyARTL	OH-R-ARTL	di-OH-ARTL
Iron(II) + DCMHQ in pyridine + ethanol	X	X	X	X	X
Iron(II) + DBMHQ in pyridine + ethanol	—	X	X	X	X
Iron(II) + SHQ in pyridine + ethanol	X	X	X	X	X
Iron(II) + SHQ in pyridine + methanol	X	X	X	X	X

Notes to Table 2
x = minor product
X = major product;
HQ = 8-Hydroxyquinoline
NHQ = 8-Hydroxy-5-nitroquinoline
CHQ = 5-Chloro-8-hydroxyquinoline
DCMHQ = 5,7-Dichloro-2-methyl-8-quinolinol
DBMHQ = 5,7-Dibromo-2-methyl-8-quinolinol
SHQ = 8-Hdroxyquinoline-5-sulfonic acid

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DQHS: R = H

AE: R = CH$_2$CH$_3$

AS: R = $\underset{\displaystyle O}{\overset{\displaystyle \|}{C}}CH_2CH_2$COOH

ARTL: R = CH$_2$ ⟶ ⟨benzene ring⟩ ⟶ COOH

FIGURE 1. Structures of dihydroqinghaosu (DQHS), arteether (AE)
artesunic acid (AS) and artelinic acid (ARTL)

Figure 2. Likely Pathways of Artelinic Acid Metabolism

[1]; ARTL

[2]; R-ARTL

[3] R = OH; OH-deoxyARTL
[4] R = H; deoxyARTL

[5]

[6] R = OH; OH-deoxyDQHS
[7] R = H; deoxyDQHS

[8]; R-DQHS

DQHS
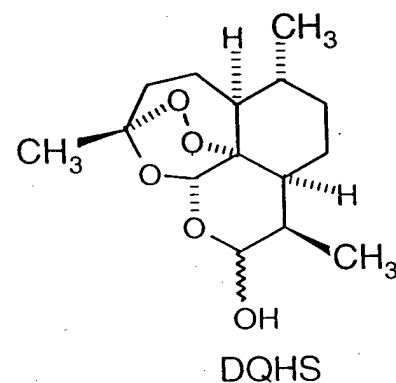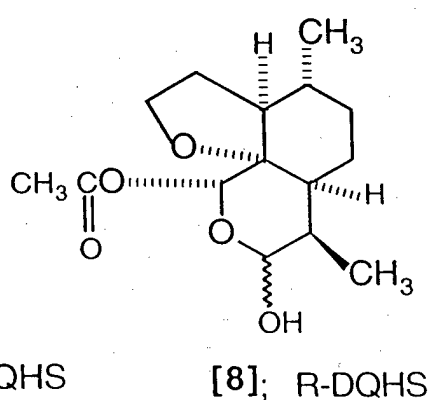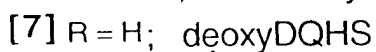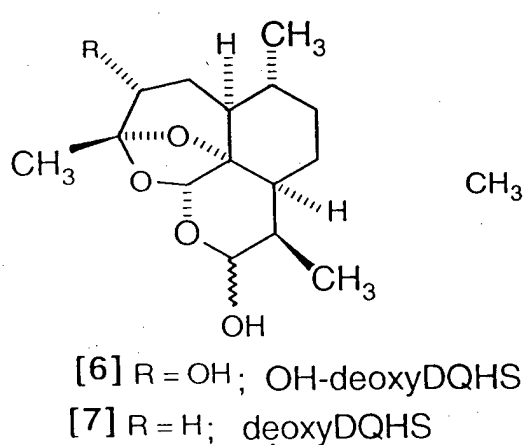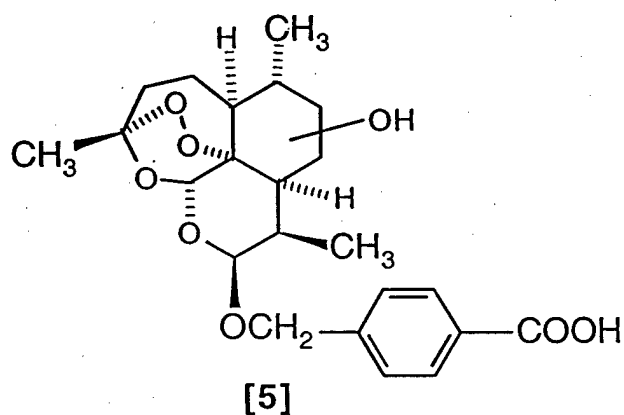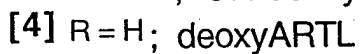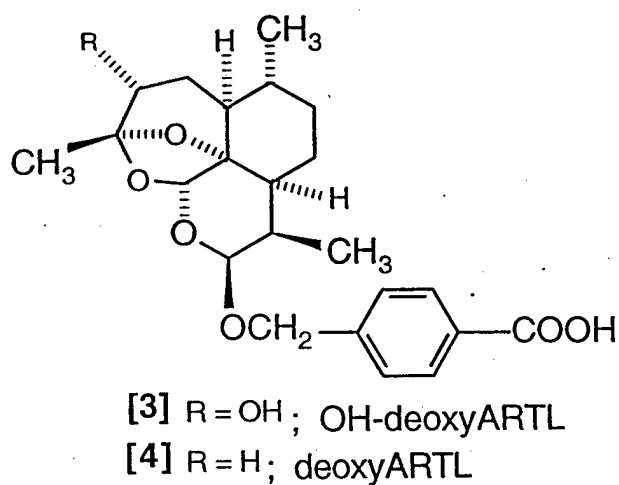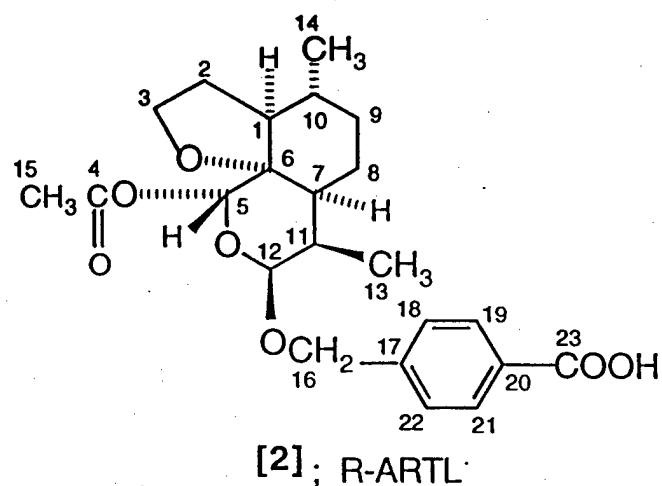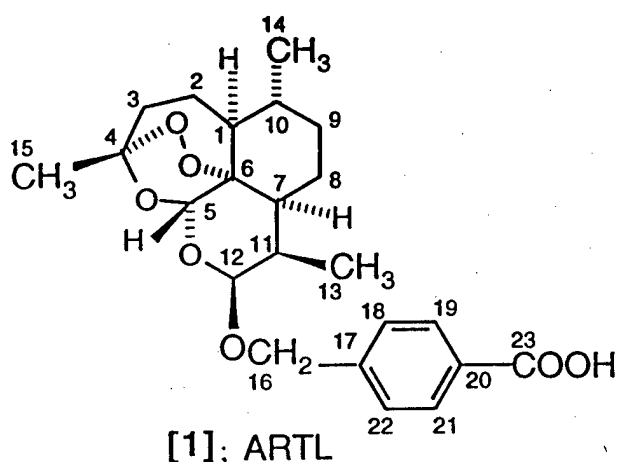
FIGURE 3: Chemical structures of artelinic acid and compounds formed from its reaction with iron(II) complexes.
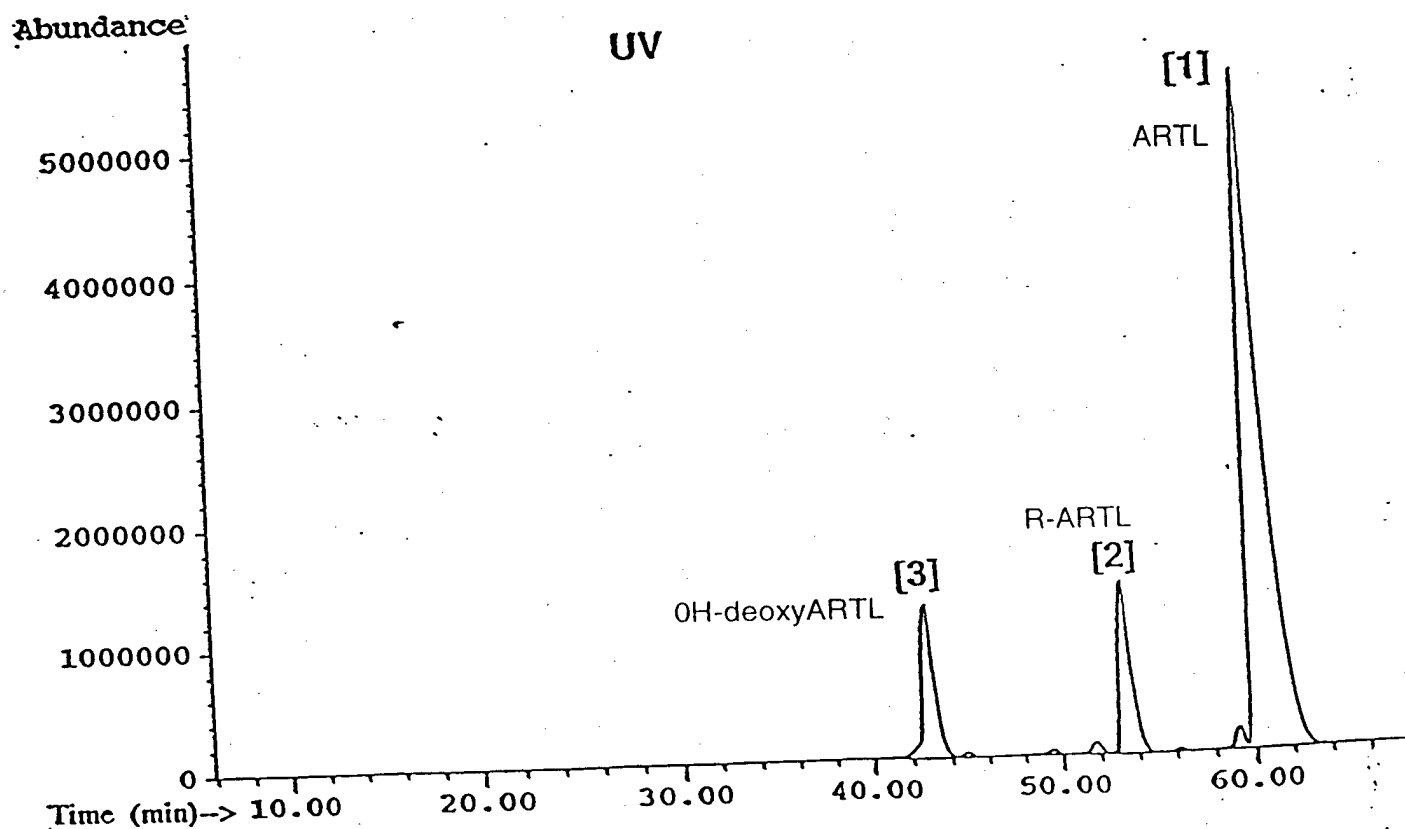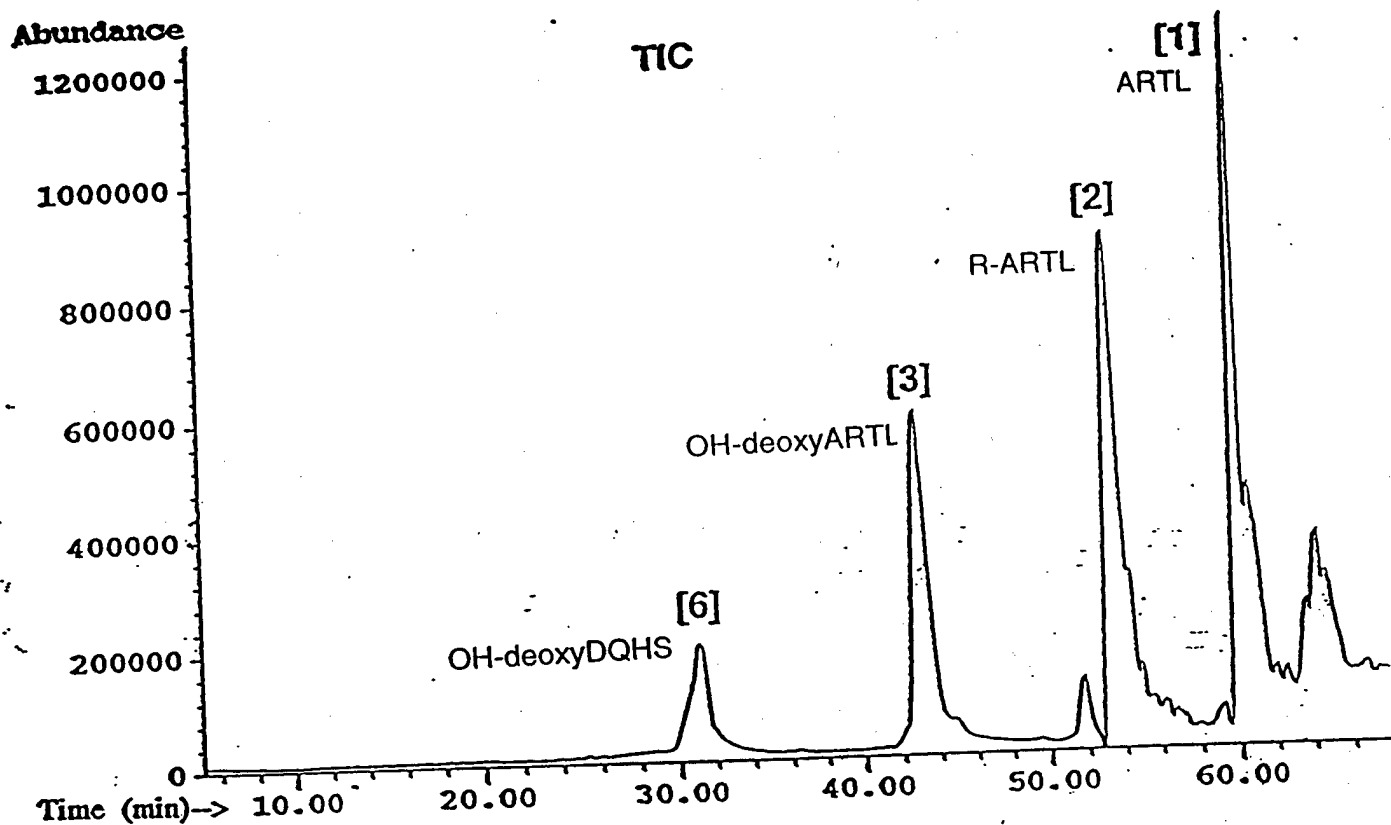
Figure 4. Total ion- and UV chromatograms of the products of the reaction of artelinic acid with the Udenfriend reaction system.
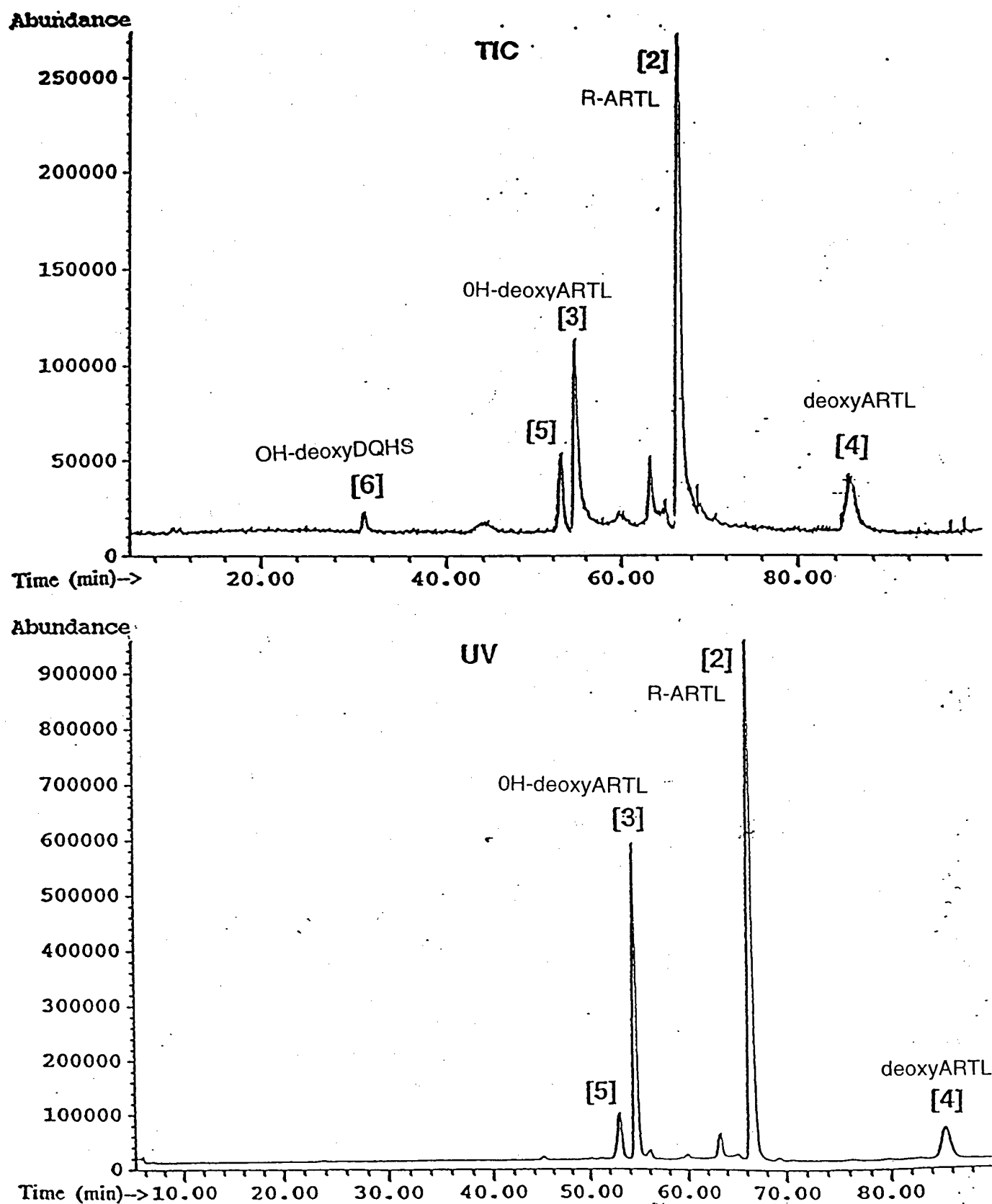
Figure 5. Total ion- and UV chromatograms of the products of the reaction of artelinic acid with the hemin-cysteine reaction system
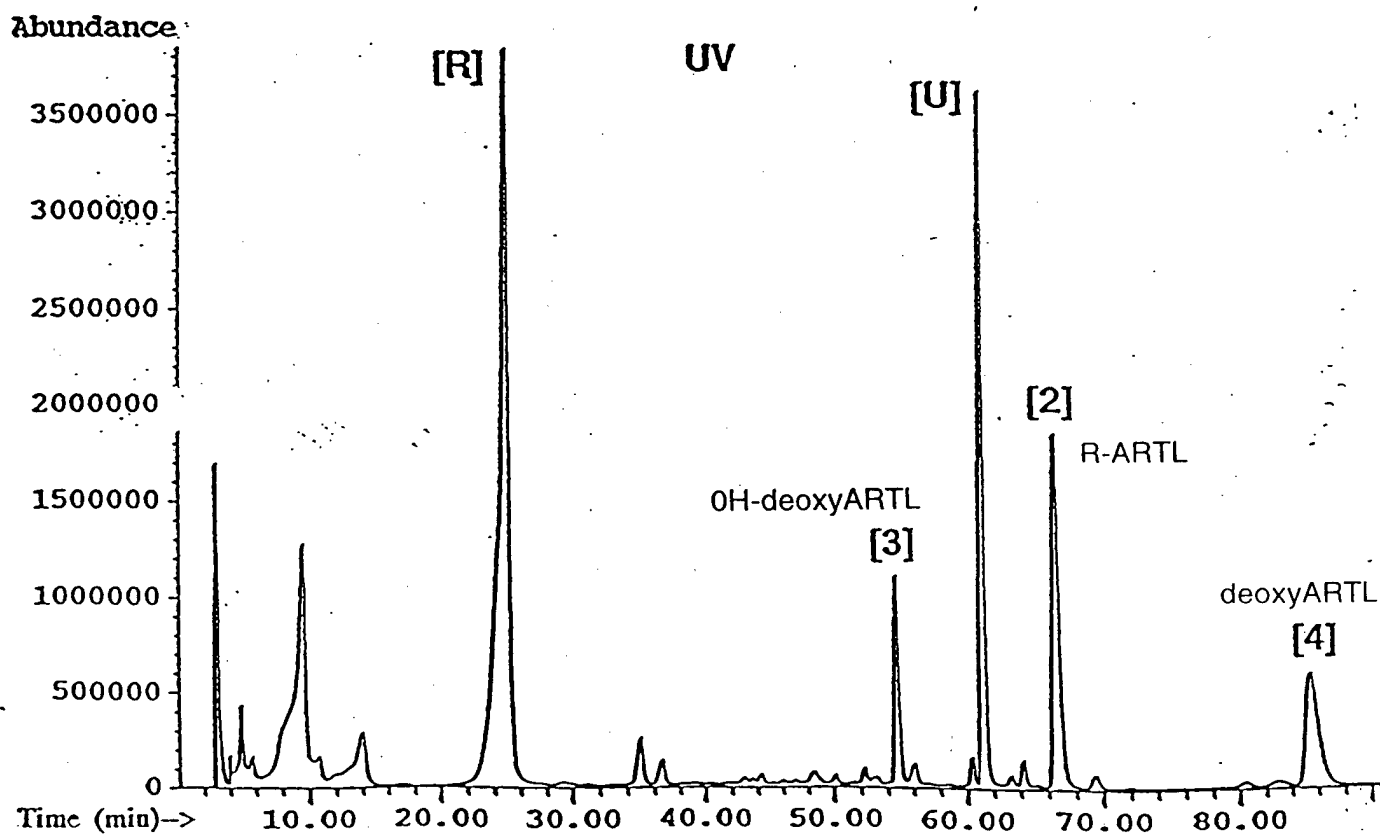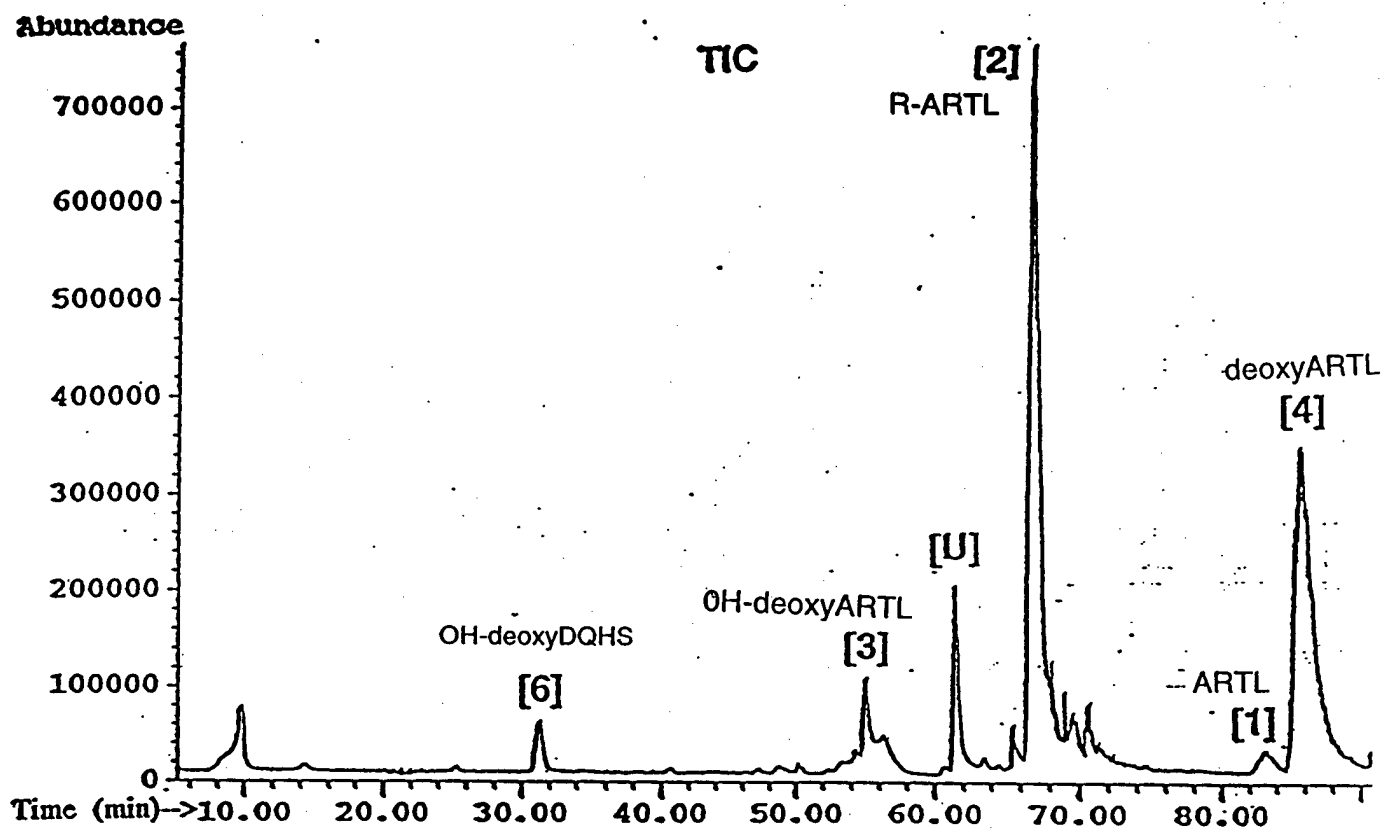
Figure 6. Total ion- and UV chromatograms of the products of the reaction of artelinic acid with the iron(II)-thiosalicylic reaction system.

Figure 7. Total ion- and UV chromatograms of the products of the reaction of artelinic acid with the iron(II)-8-hydroxyquinoline reaction system.

Figure 8. 13C-nmr spectrum of artelinic acid.

Figure 9. 1H-nmr spectrum of artelinic acid.

Figure 10. Thermospray mass spectrum of artelinic acid.

Figure 11. Proposed fragmentation pattern for the thermospray mass spectrum of artelinic acid.

Figure 12. 1H-nmr spectrum of rearranged artelinic acid (R-ARTL)
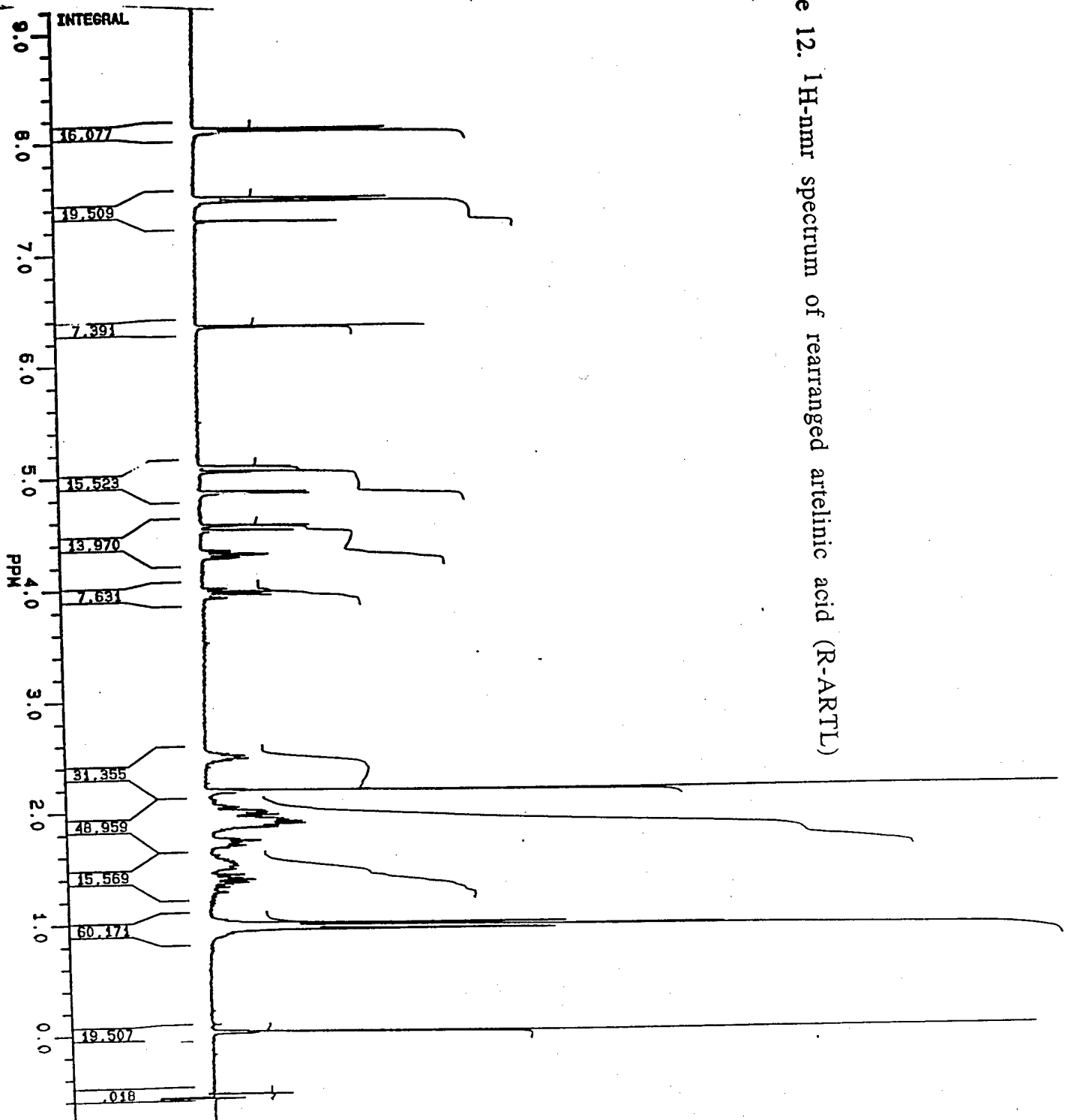
Figure 13. 13C-nmr spectrum of rearranged artelinic acid (R-ARTL).
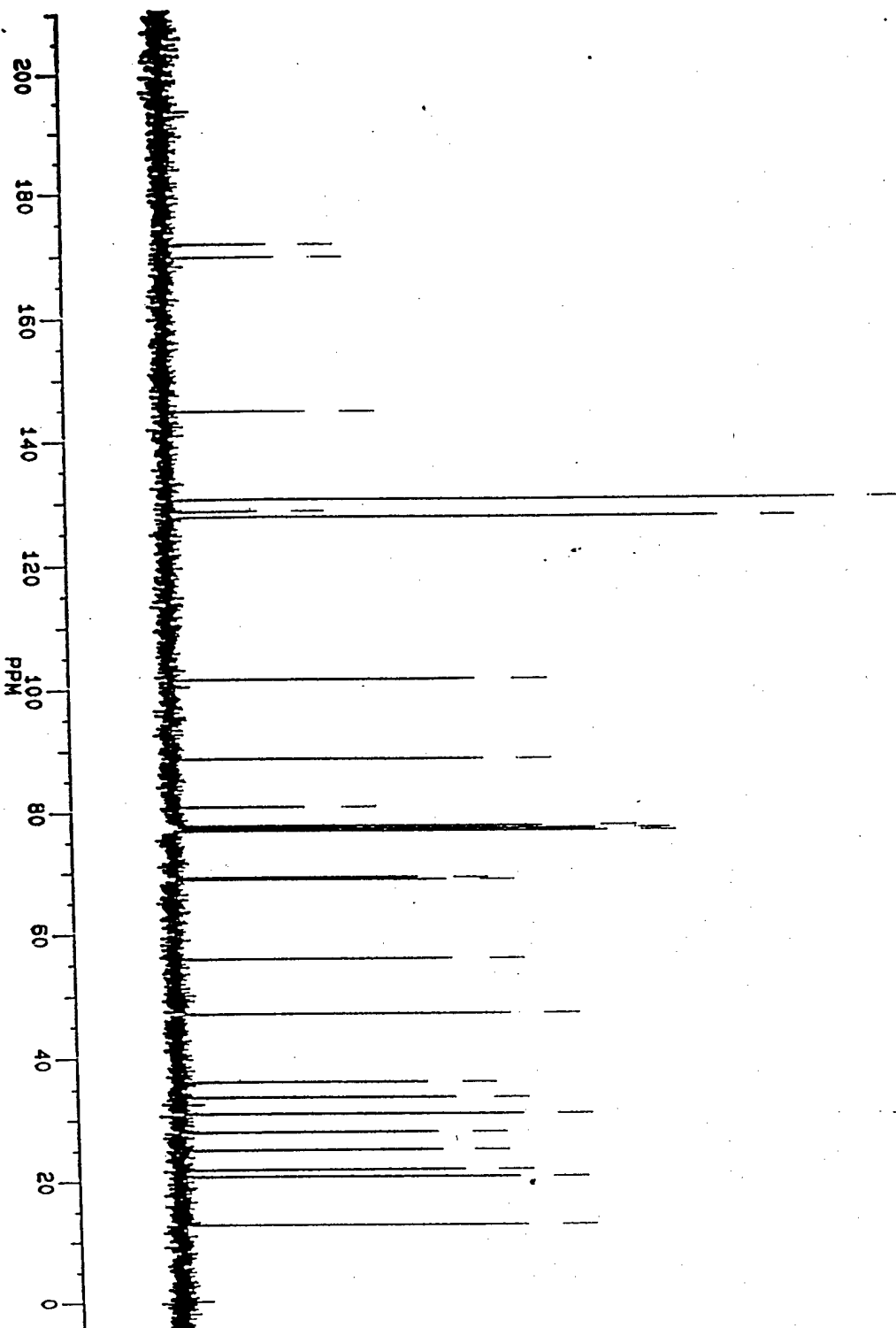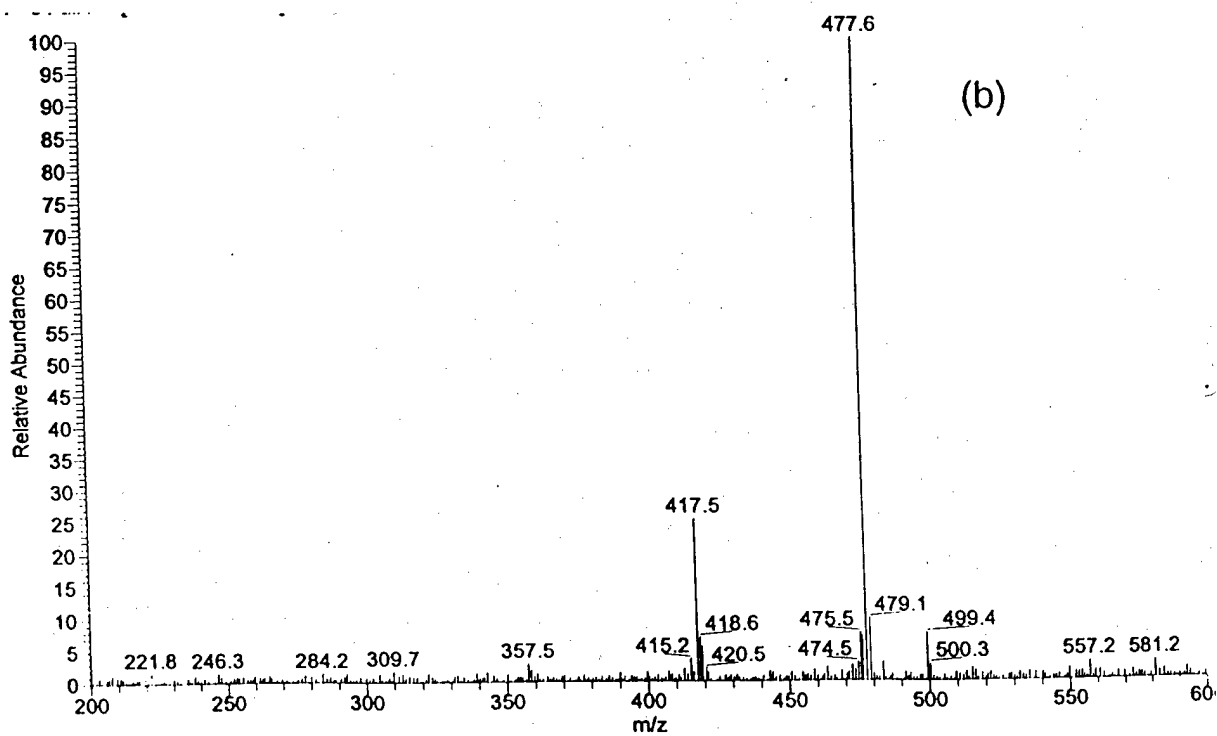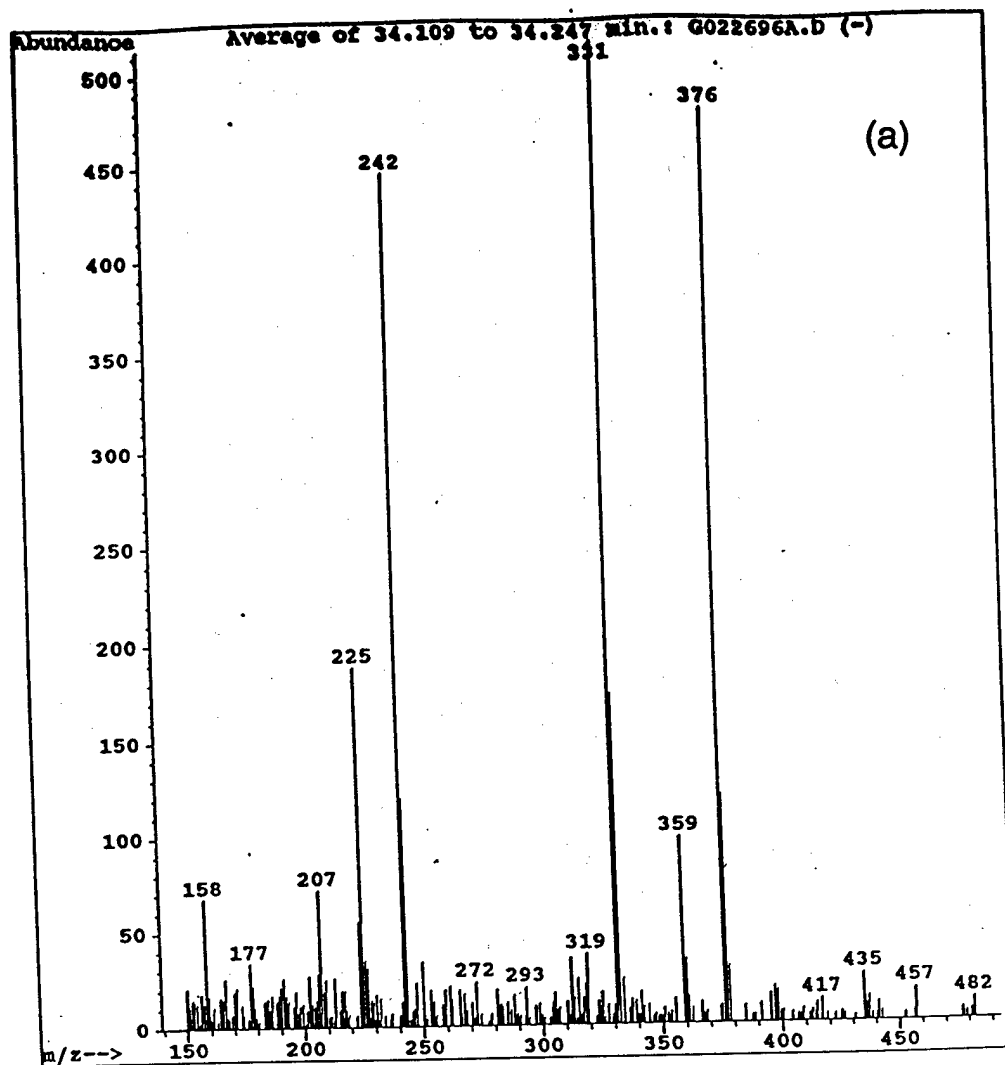
Figure 14. (a) Thermospray mass spectrum and (b) Negative ion mass spectrum of rearranged artelinic acid (R-ARTL).
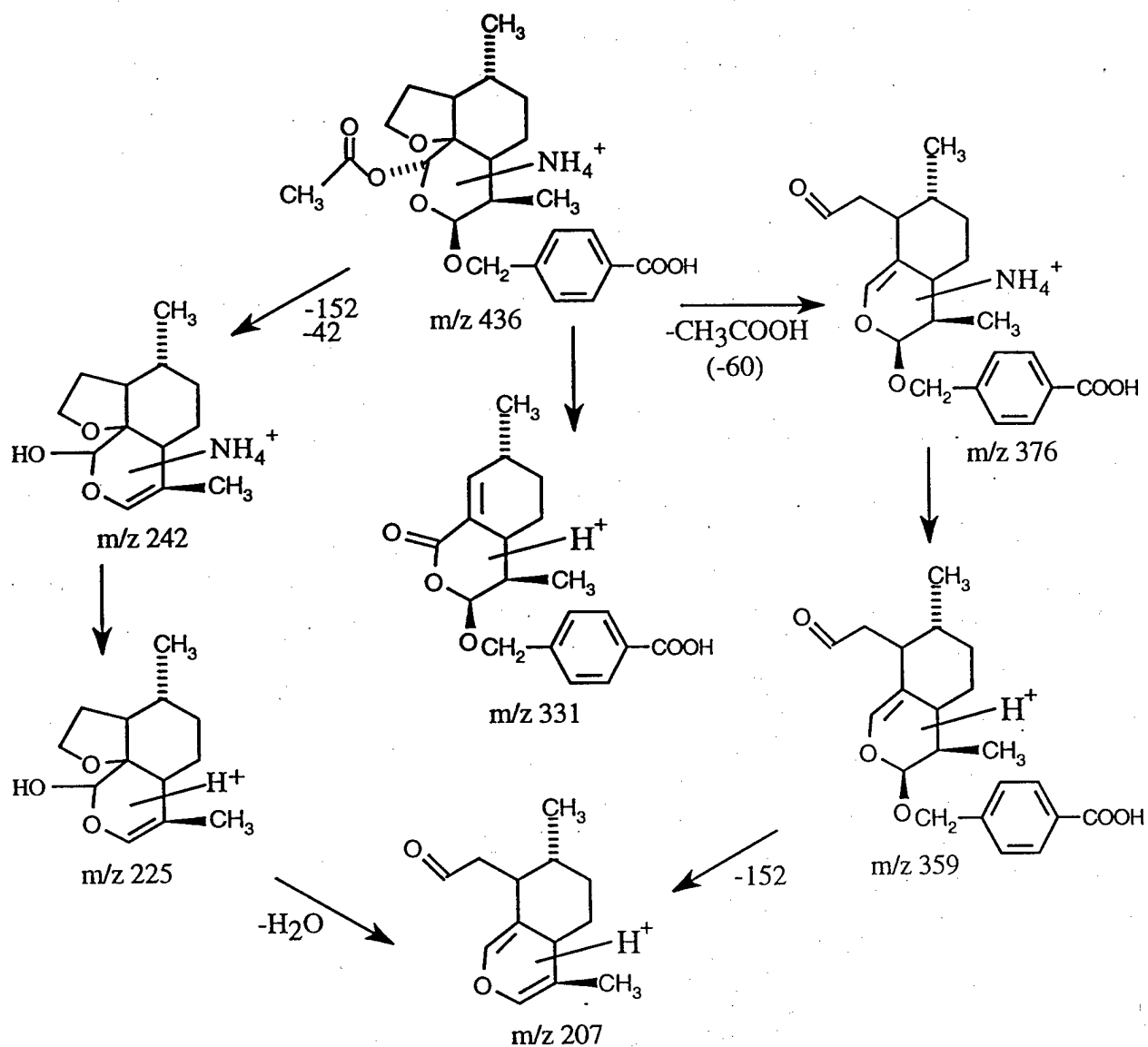
Figure 15. Proposed fragmentation pattern for the thermospray mass spectrum of rearranged artelinic acid (R-ARTL)

Figure 16. 1H-nmr spectrum of hydroxydeoxyyartelinic acid (OH-deoxyARTL).
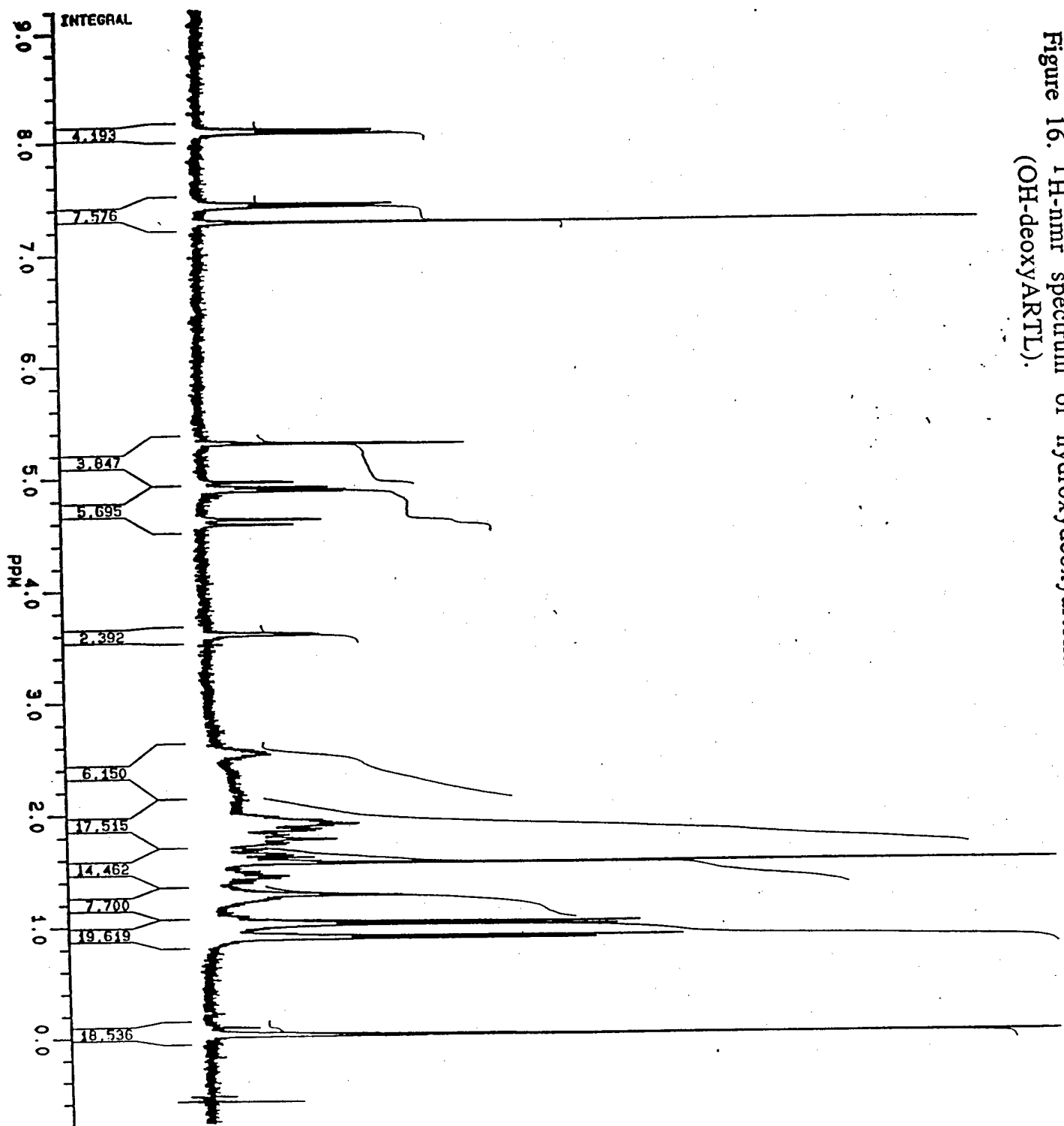
Figure 17. 13C-nmr spectrum of hydroxydeoxyyartelinic acid (OH-deoxyARTL).
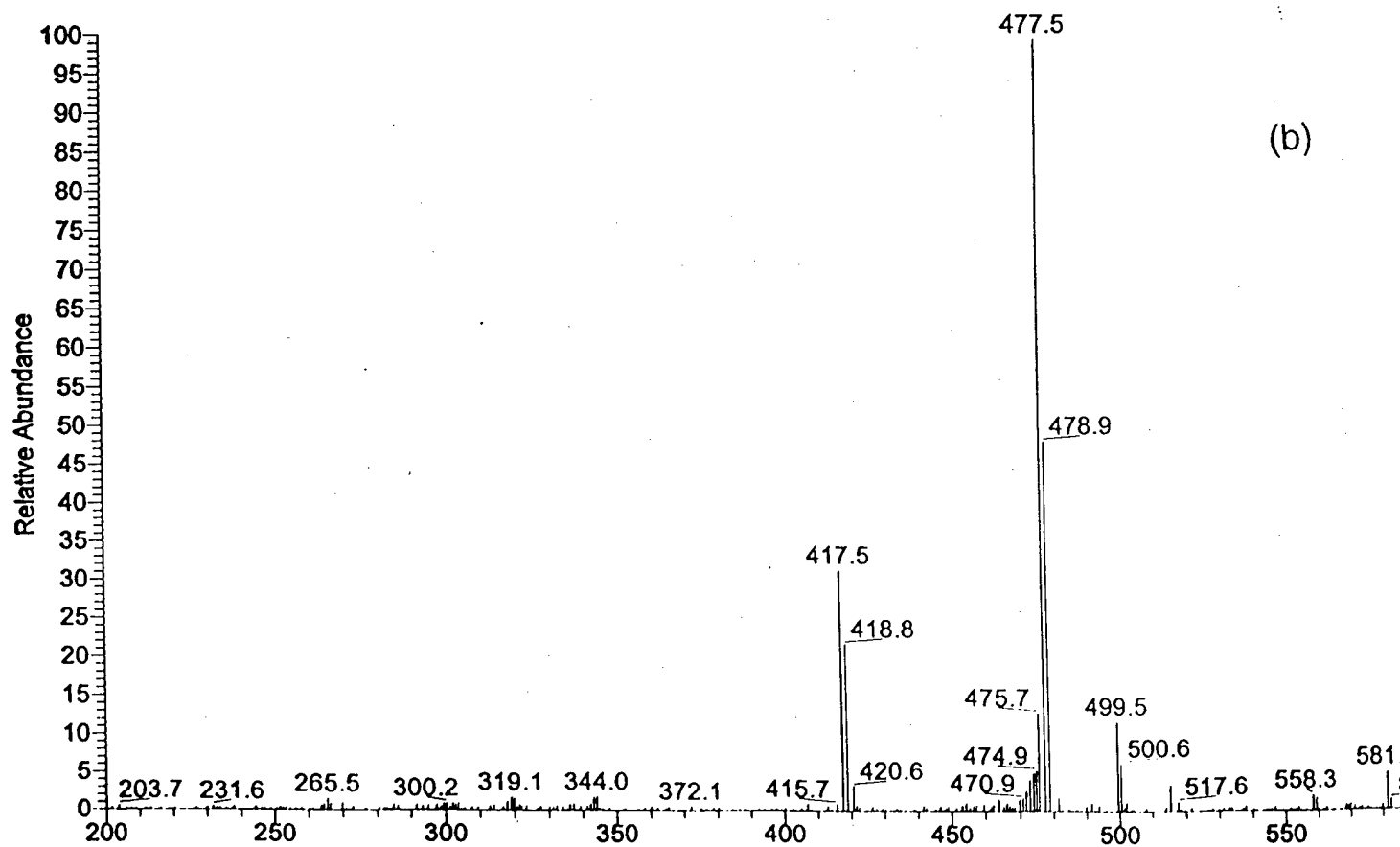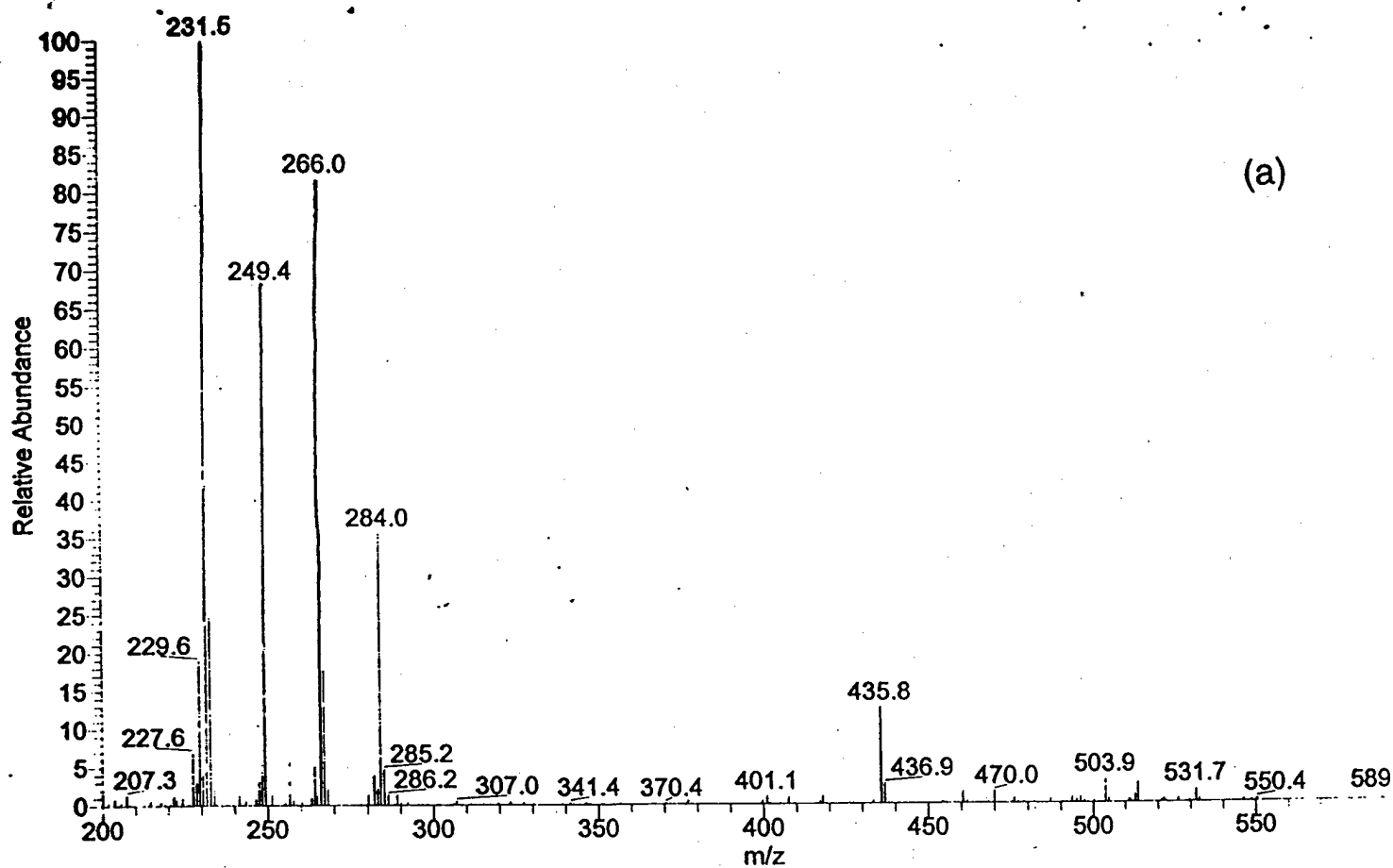
Figure 18. (a) Thermospray mass spectrum and (b) Negative ion mass spectrum of 3-hydroxydeoxyartelinic acid (OH-deoxyARTL).
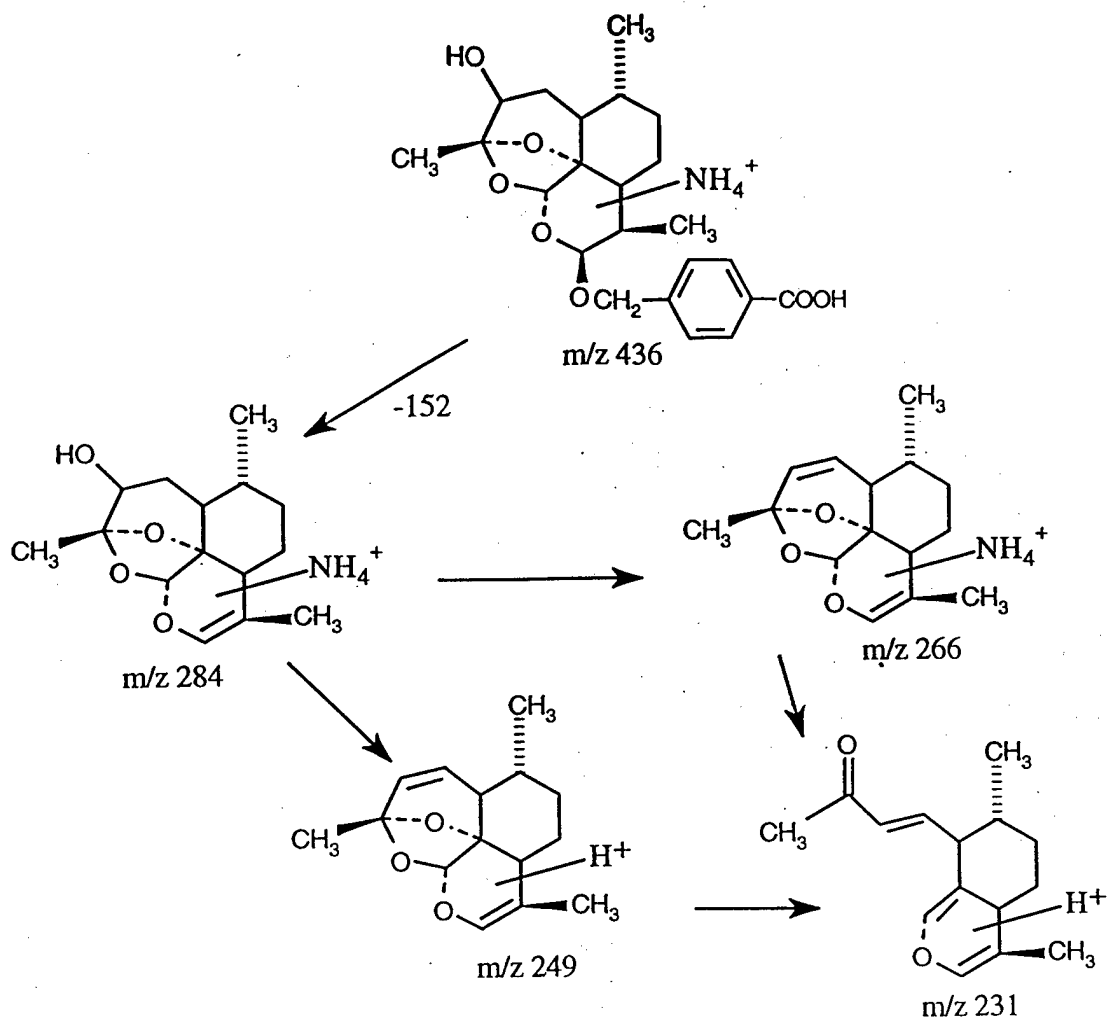
Figure 19. Proposed fragmentation pattern for the thermospray mass spectrum of 3-hydroxydeoxyartelinic acid

Figure 20. 1H-nmr spectrum of deoxyartelinic acid (deoxyARTL).
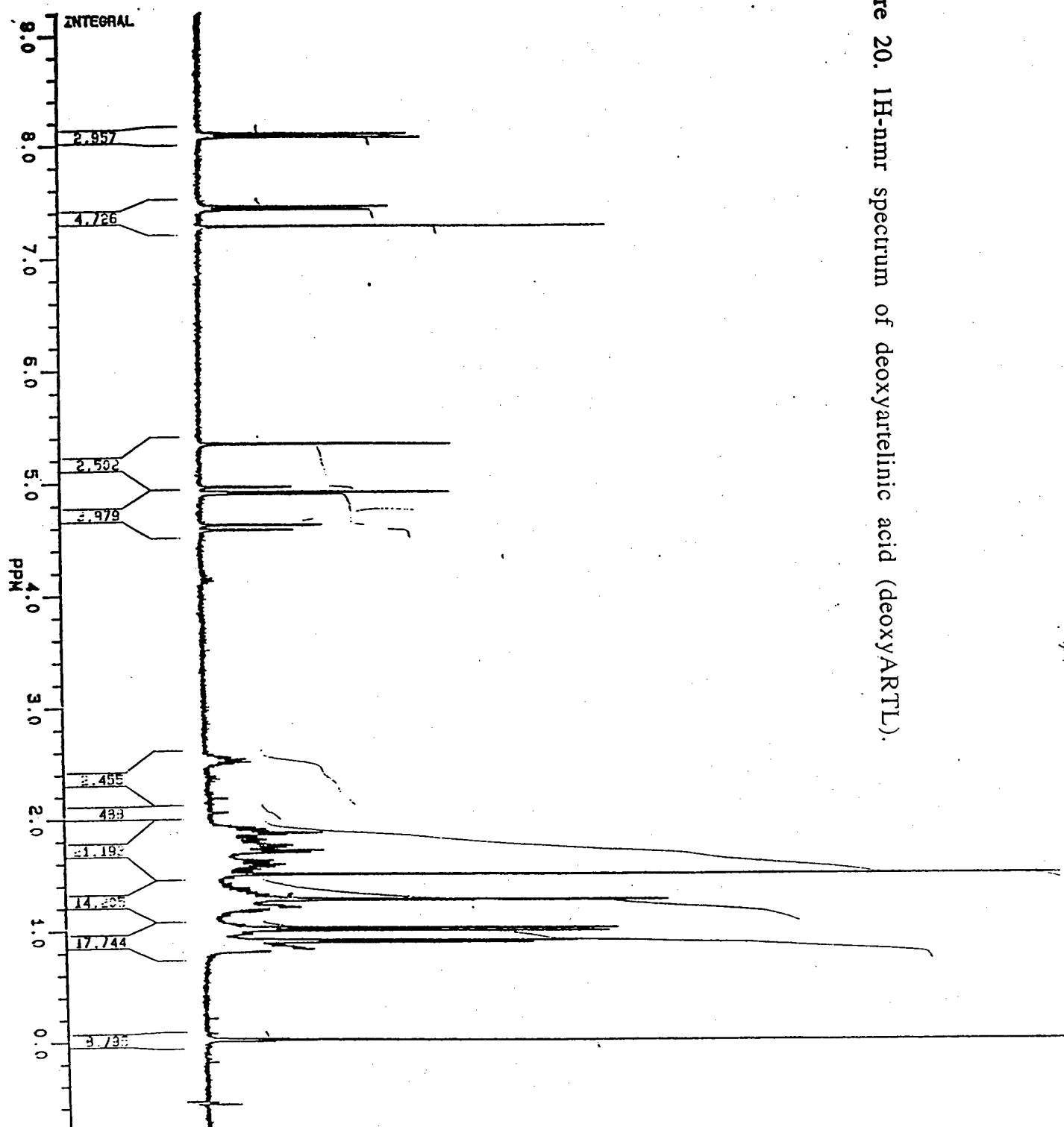
Figure 21. 13C-nmr spectrum of deoxyartelinic acid (deoxyARTL).
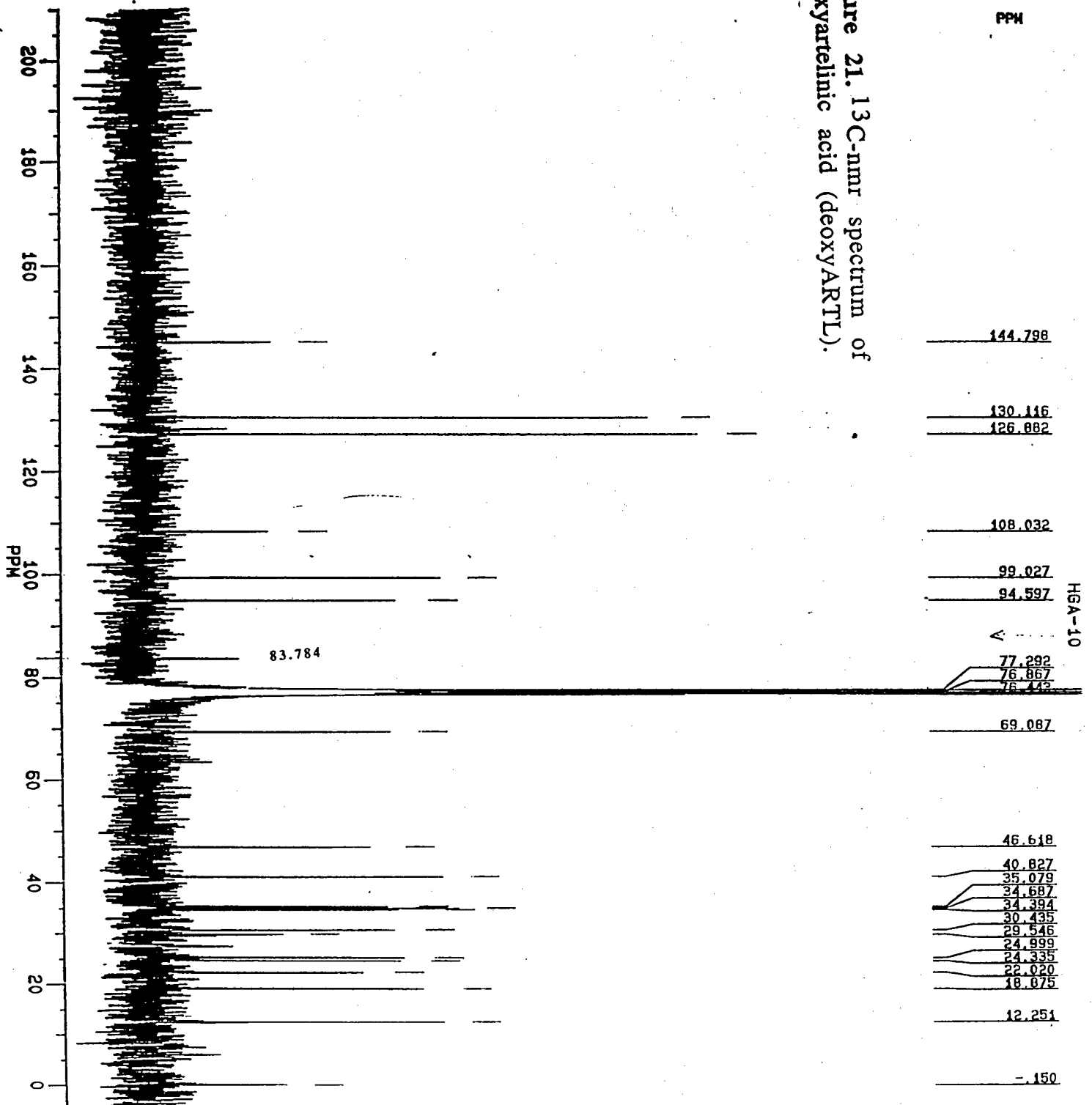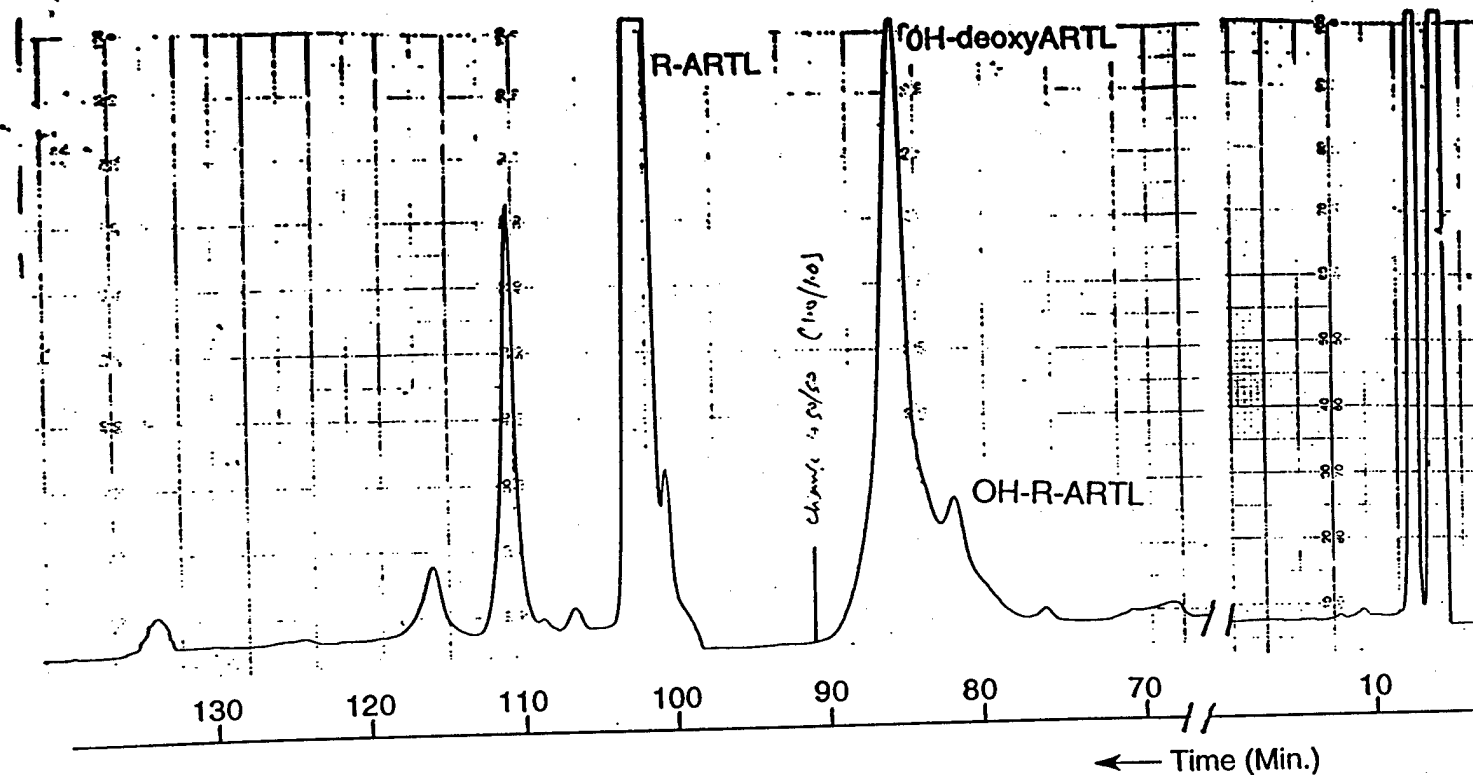
Figure 22. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxyquinoline
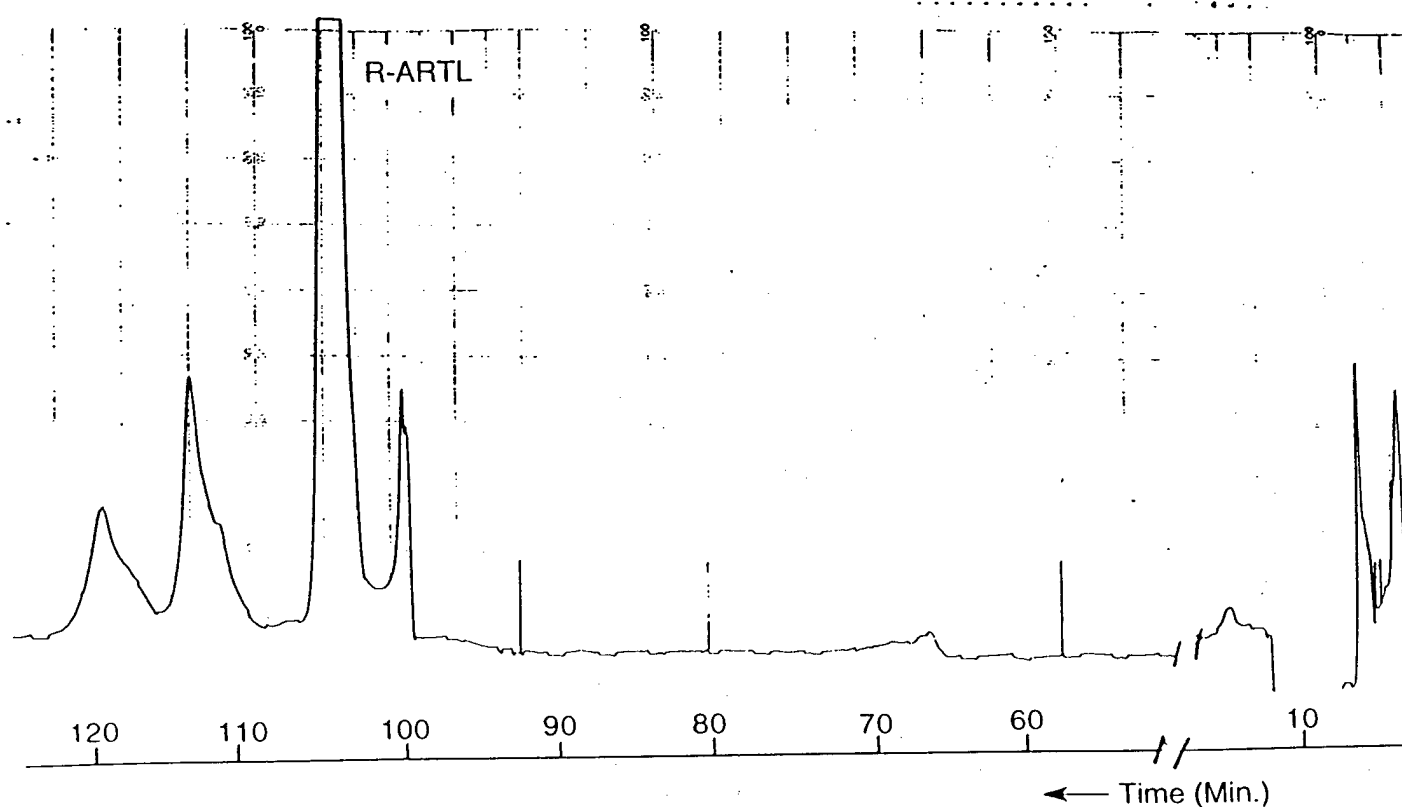
Figure 23. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) sulfate and 8-hydroxyquinoline.
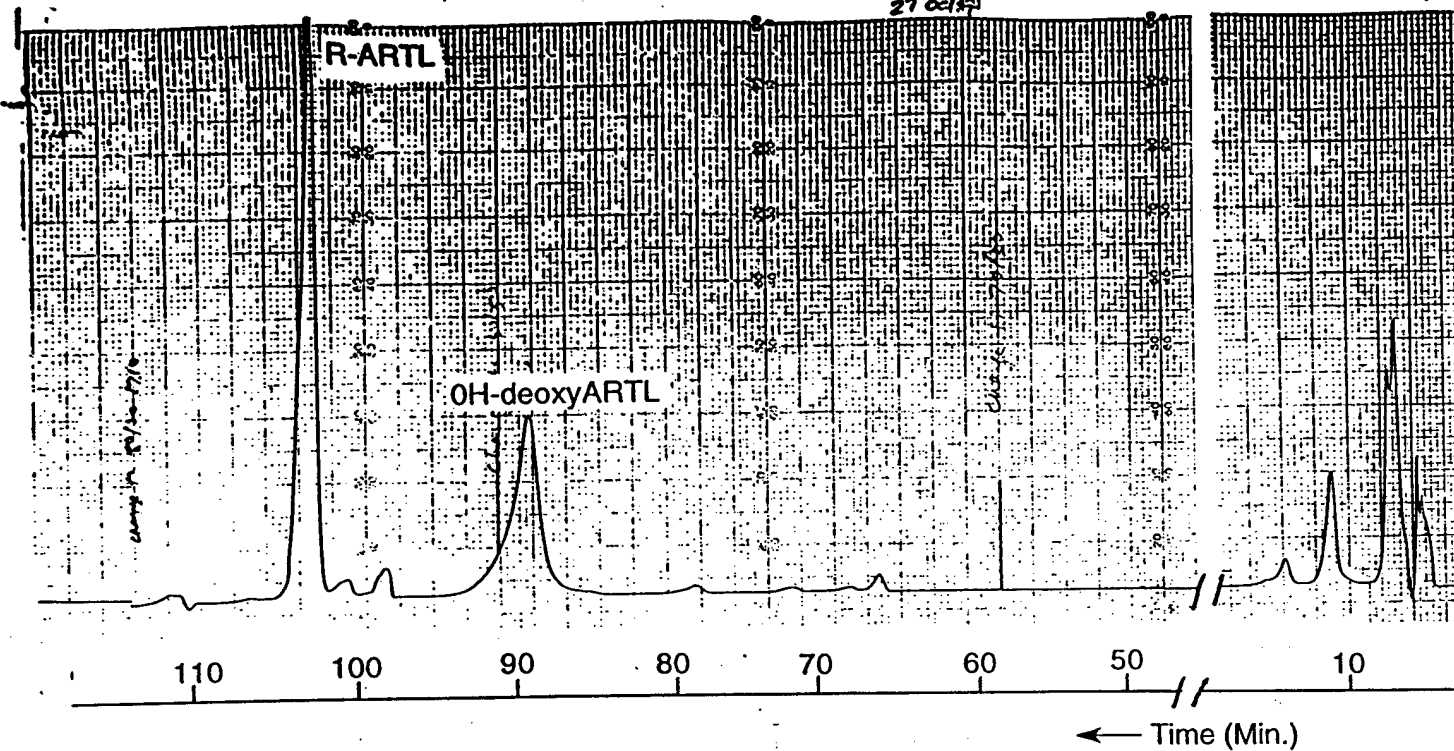
Figure 24. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
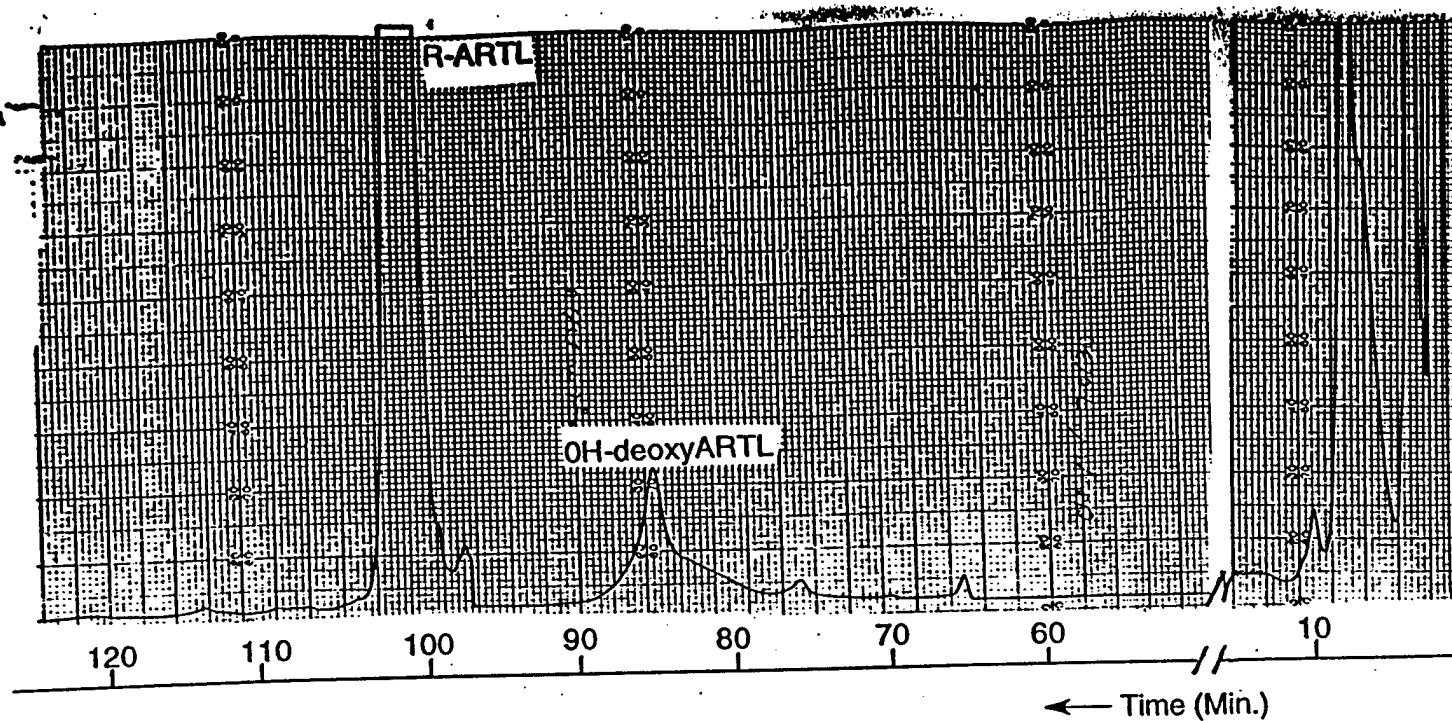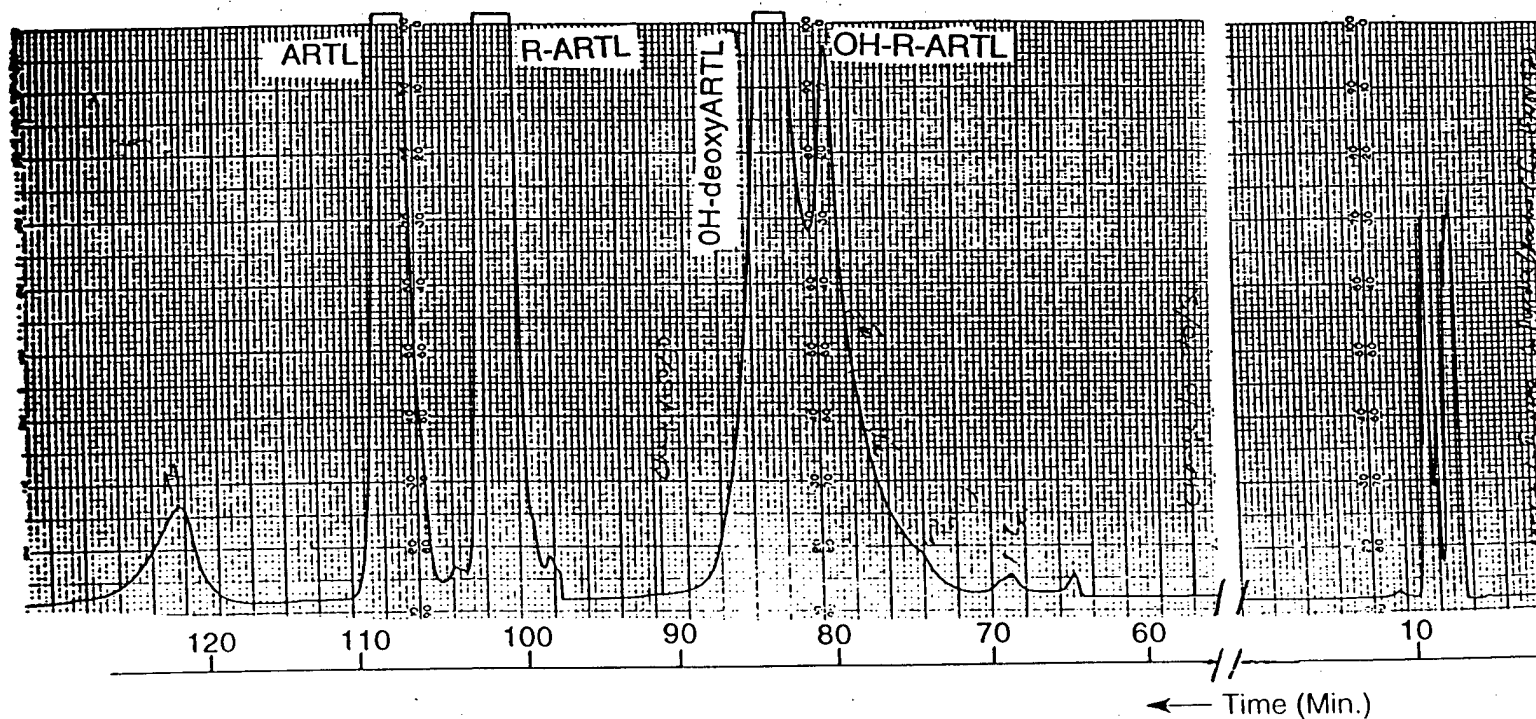8-hydroxyquinoline in a mixture of acetone and
mercaptoethanol.
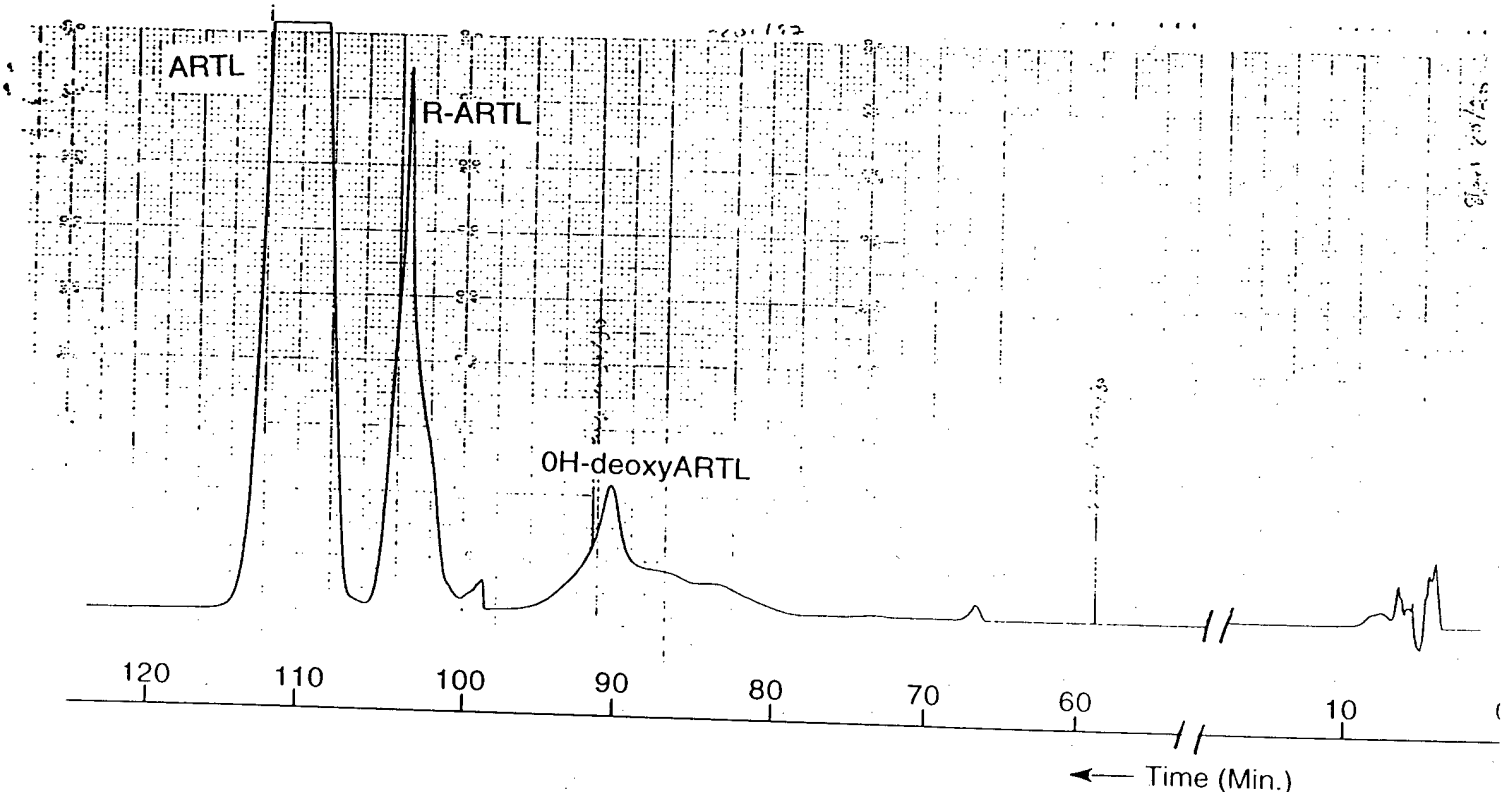
Figure 25. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
8-hydroxyquinoline in a mixture of acetone and pyridine.
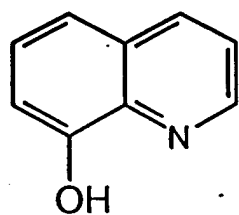
8-Hydroxyquinoline (HQ)
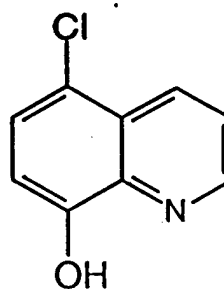
5-Chloro-8-hydroxyquinoline (CHQ)
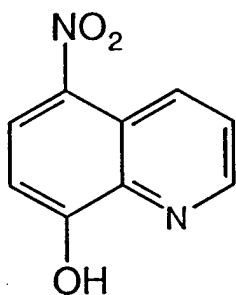
8-Hydroxy-5-nitroquinoline (NHQ)
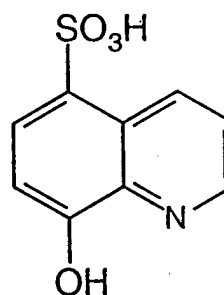
8-Hydroxyquinoline-5-sulfonic acid (SHQ)
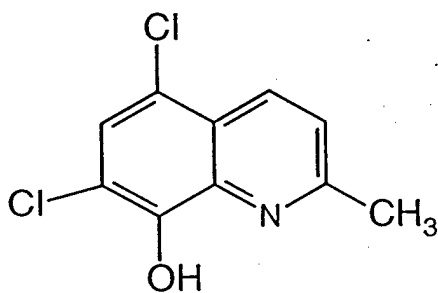
5,7-Dichloro-2-methyl-8-quinolinol (DCMHQ)
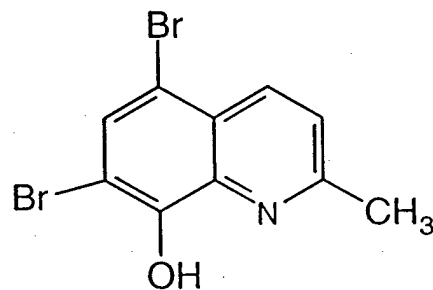
5,7-Dibromo-2-methyl-8-quinolinol (DBMHQ)

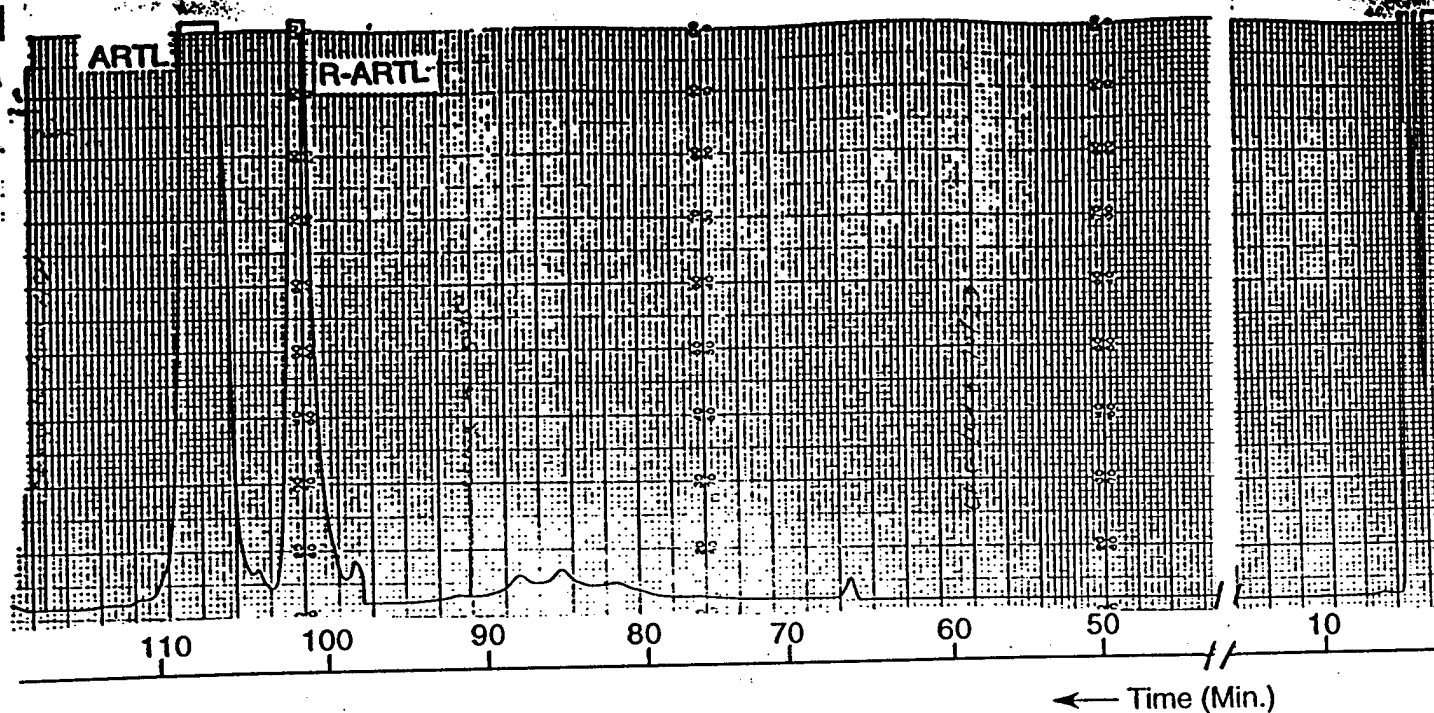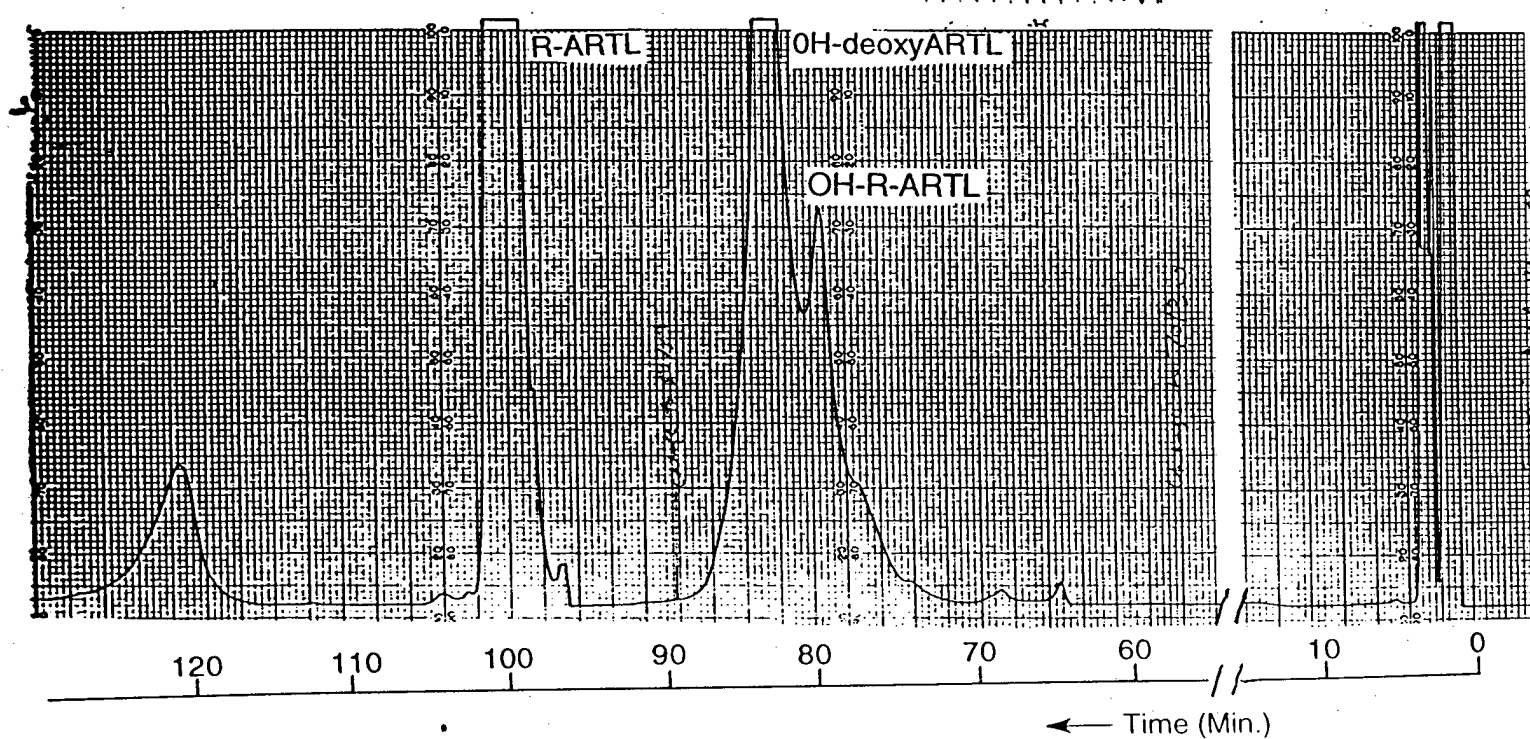FIGURE 26. Chemical structures of 8-hydroxyquinoline
and its substituted analogs

Figure 27. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of acetone and mercaptoethanol.

Figure 28. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxy-5-nitroquinoline (NHQ) in a mixture of acetone and pyridine.

Figure 29. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxy-5-nitroquinoline (NHQ) in pure acetone.

Figure 30. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 8-hydroxy-5-nitroquinoline (NHQ) in pure pyridine.
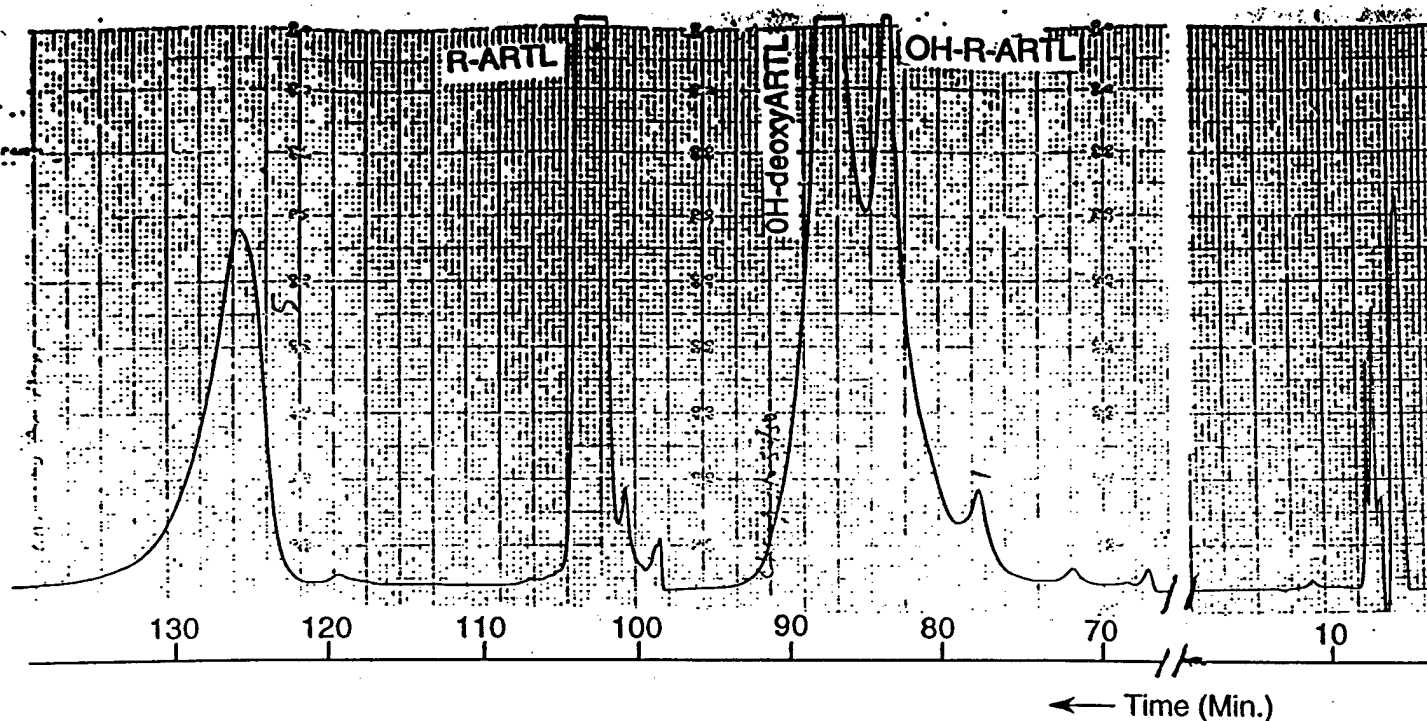
Figure 31. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
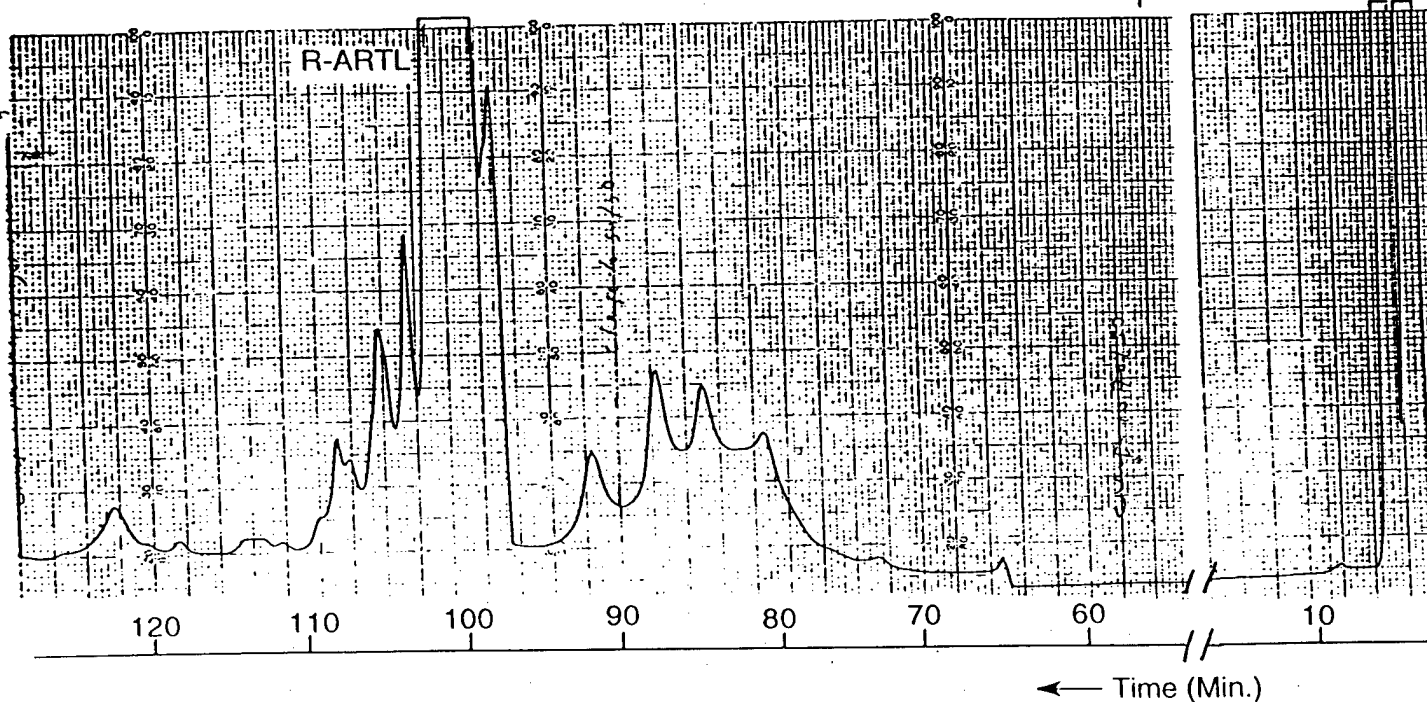8-hydroxy-5-nitroquinoline (NHQ) in a dichloromethane
containing imidazole.

Figure 32. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
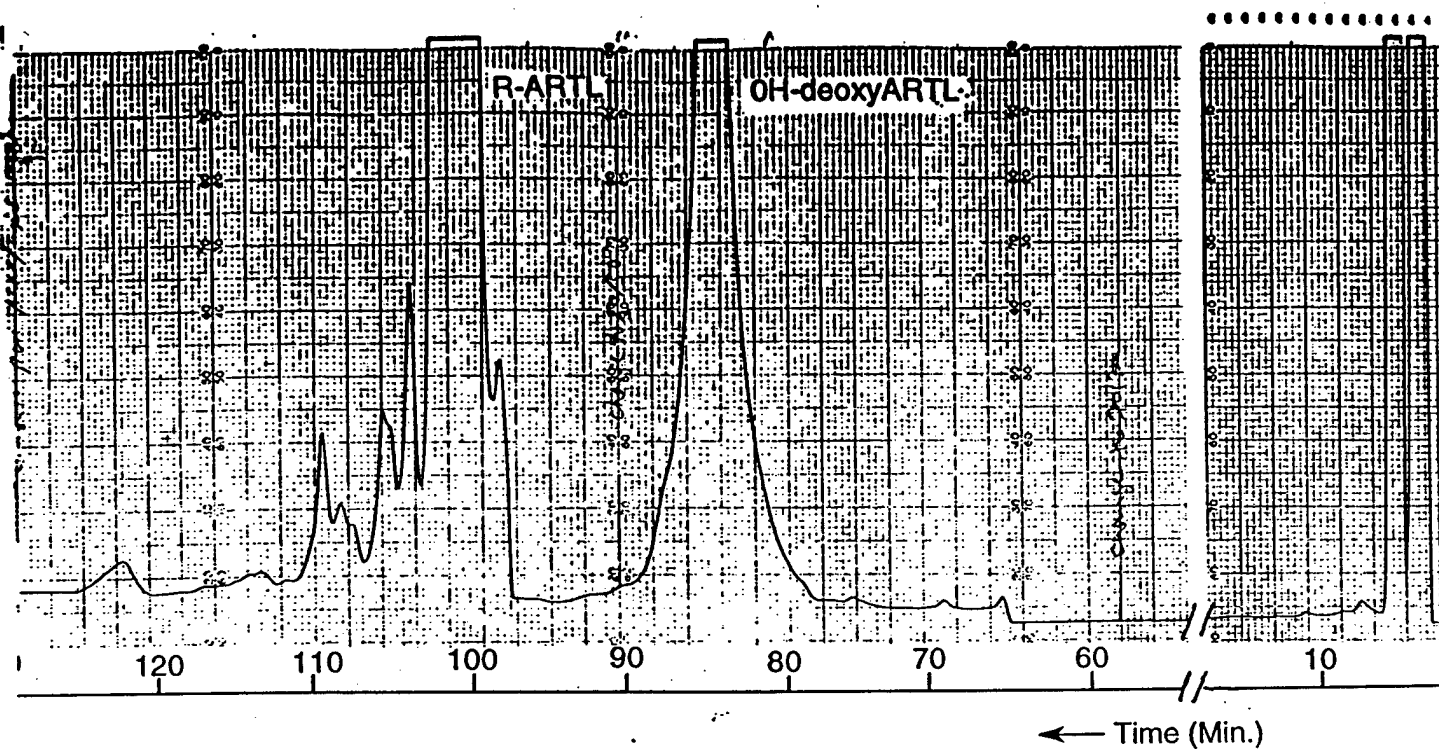8-hydroxy-5-nitroquinoline (NHQ) pure dichloromethane.

Figure 33. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
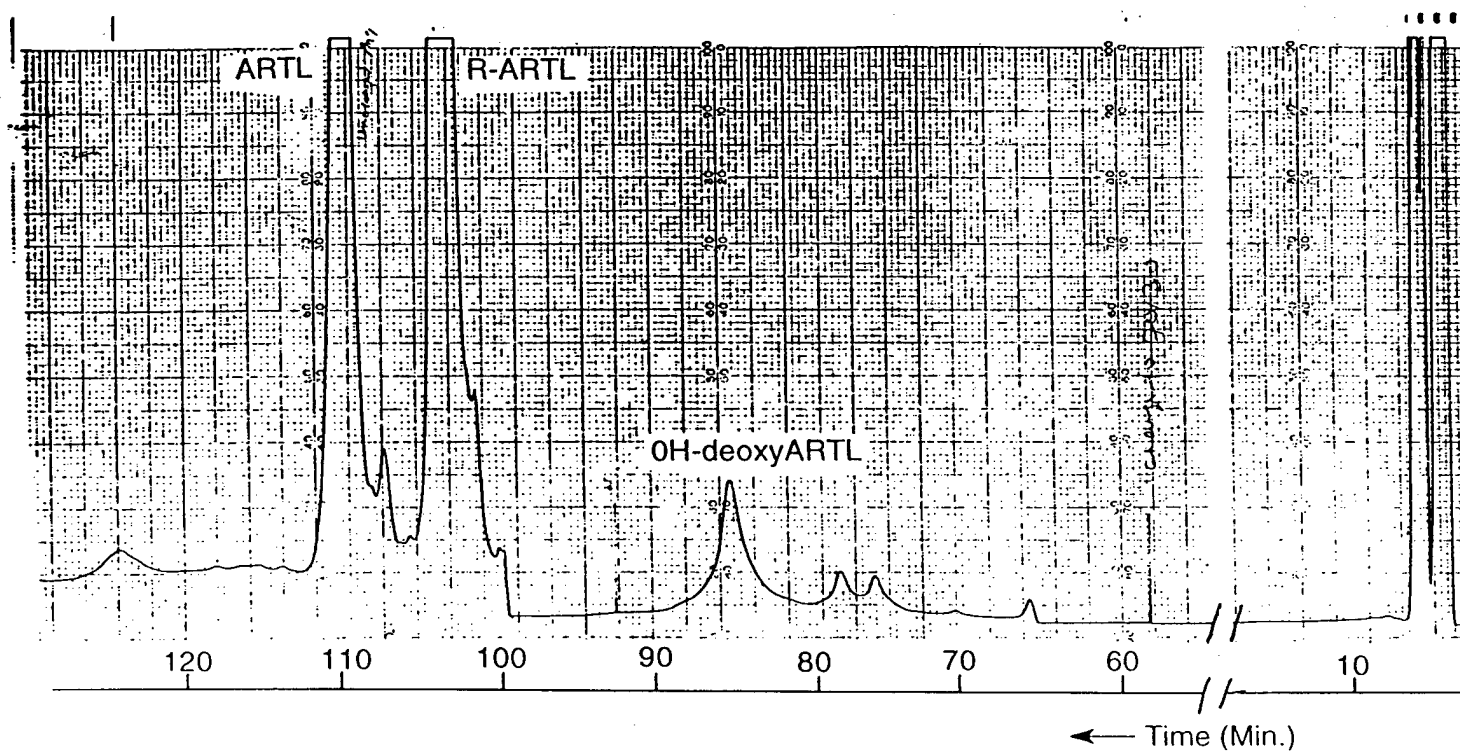8-hydroxy-5-nitroquinoline (NHQ) in pure benzene.

Figure 34. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
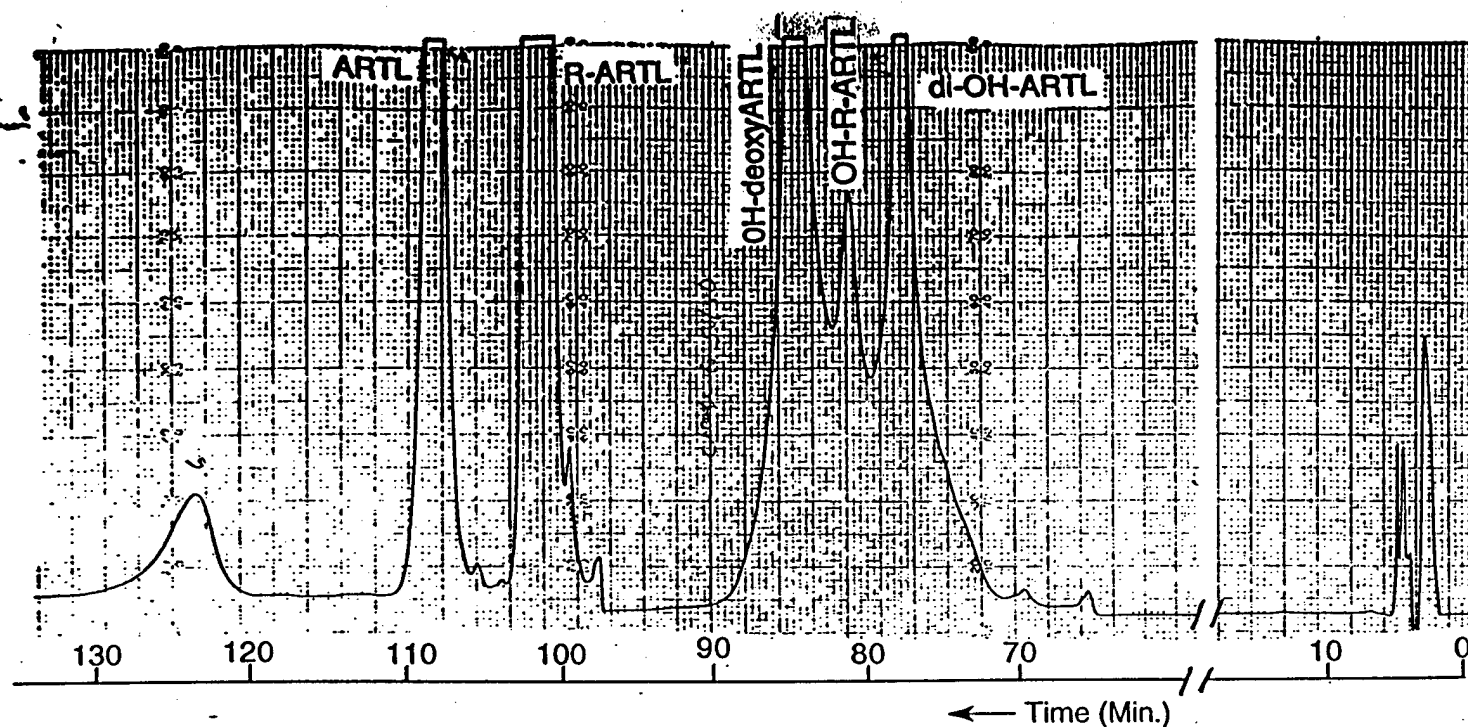8-hydroxy-5-nitroquinoline (NHQ) in absolute ethanol.

Figure 35. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
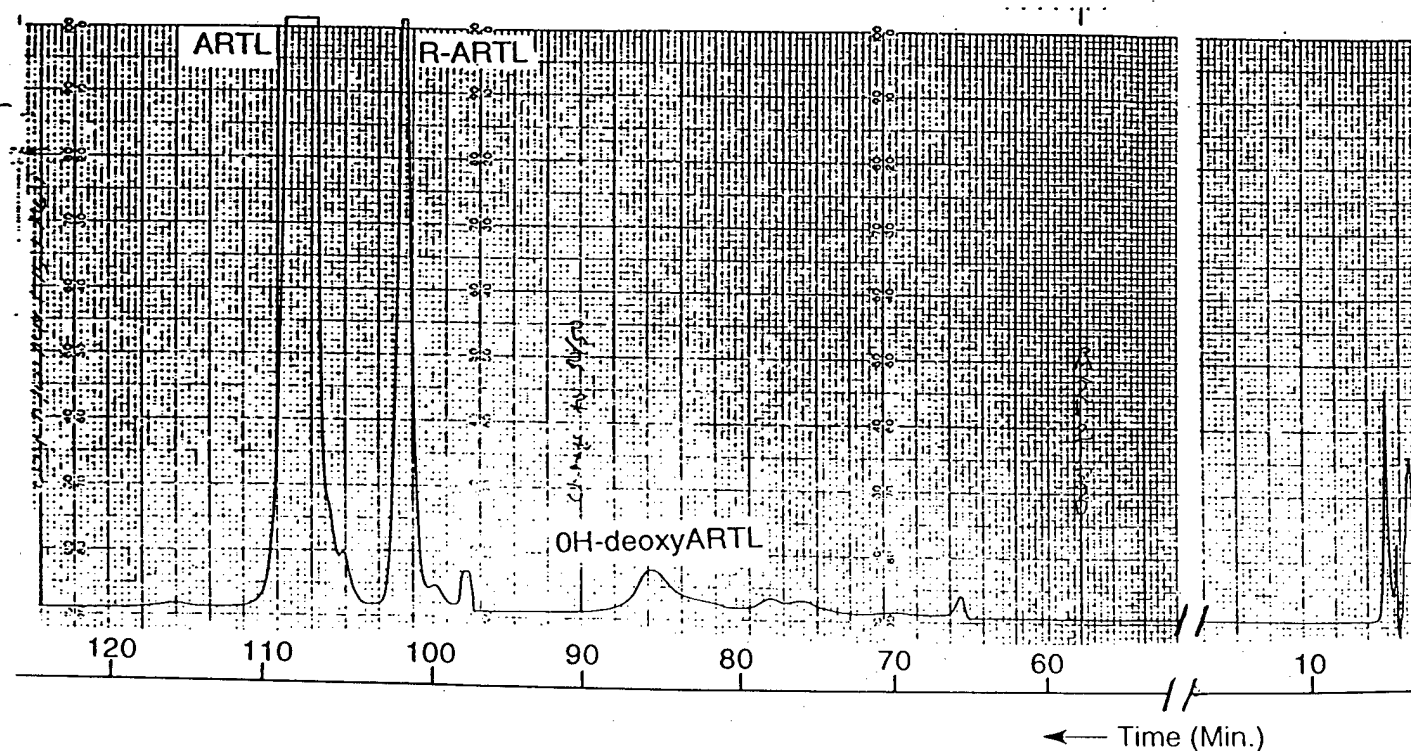8-hydroxy-5-nitroquinoline (NHQ) in a mixture of pyridine
and ethanol.

Figure 36. HPLC chromatogram of the products of the reaction of
artelinic acid with iron(II) chloride and
8-hydroxyquinoline (HQ) in a mixture of pyridine and
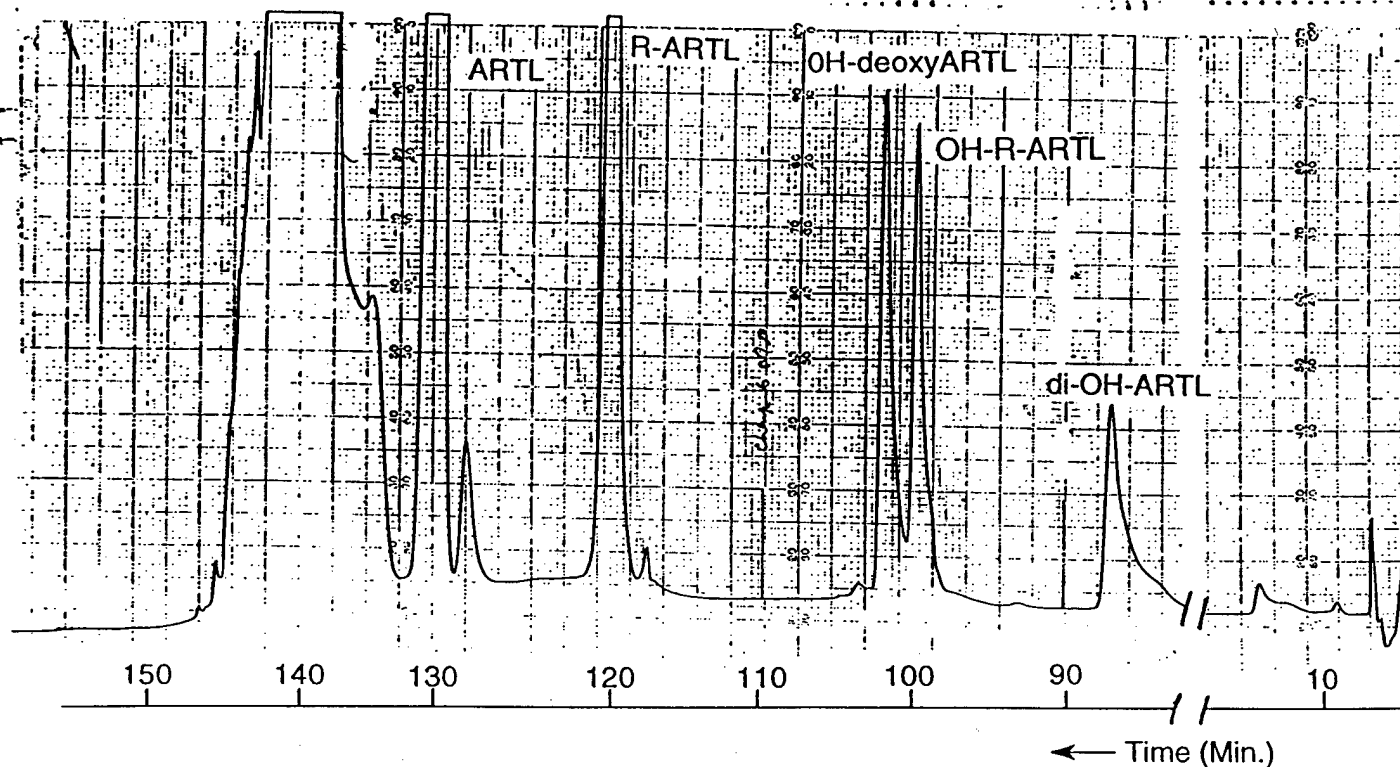ethanol.

Figure 37. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II) chloride and 5,7-dichloro-2-methyl-8-quinolinol (DCMHQ) in a mixture of pyridine and methanol.
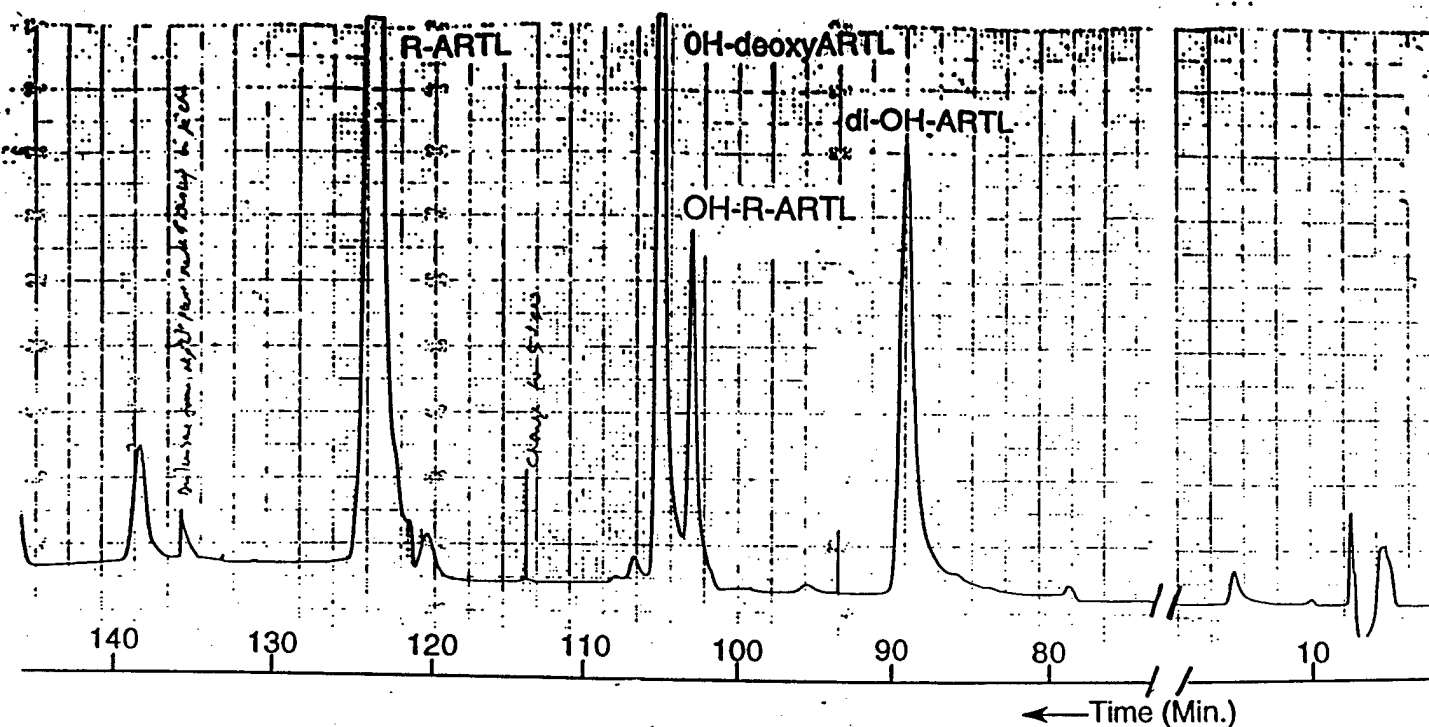
Figure 38. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II)chloride and 5,7-dibromo-2-methyl-8-quinolinol (DBMHQ) in a mixture of pyridine and methanol.
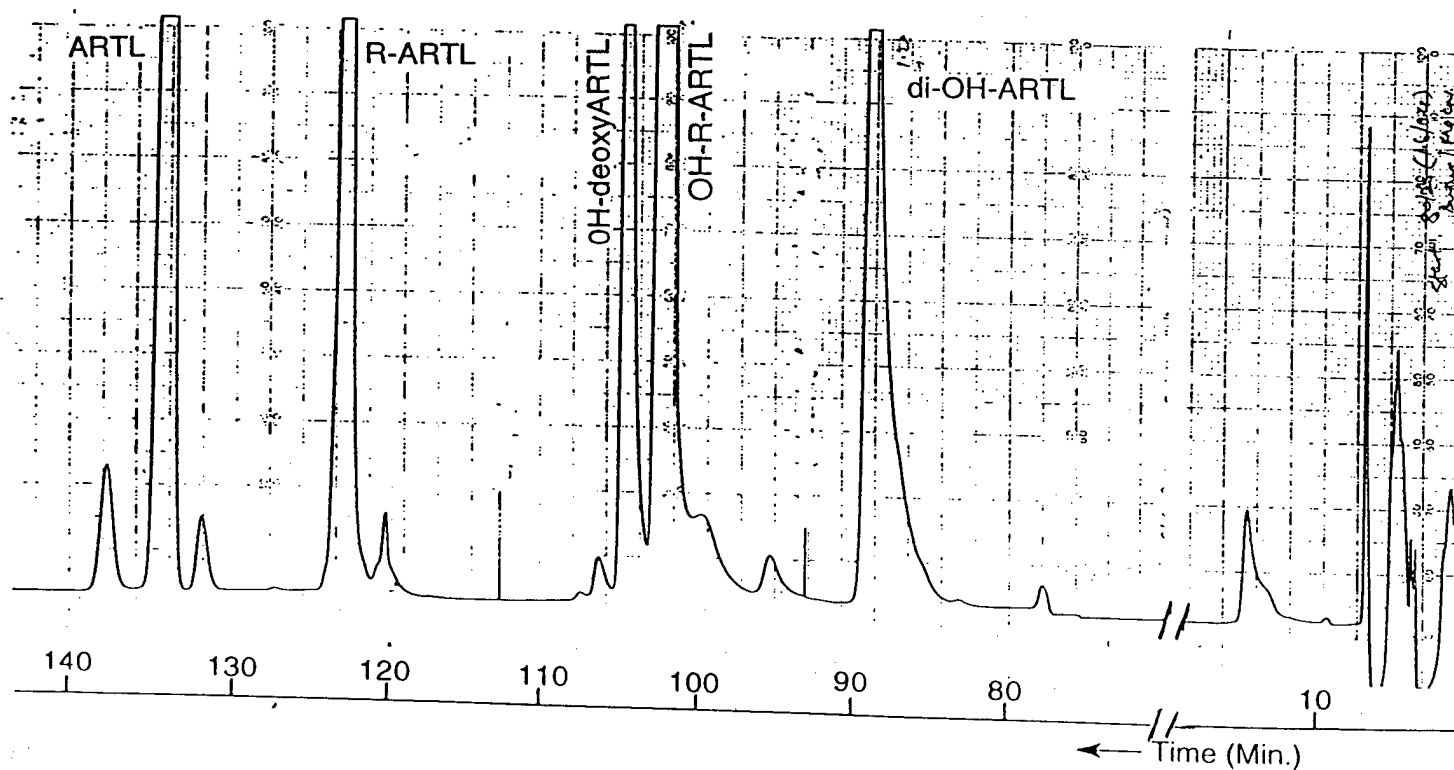
Figure 39. HPLC chromatogram of the products of the reaction of artelinic acid with iron(II)chloride and 8-hydroxyquinoline-5-sulfonic acid (SHQ) in a mixture of pyridine and methanol.

Figure 40. 1H-nmr spectrum of hydroxylated rearranged artelinic acid (OH-R-ARTL).
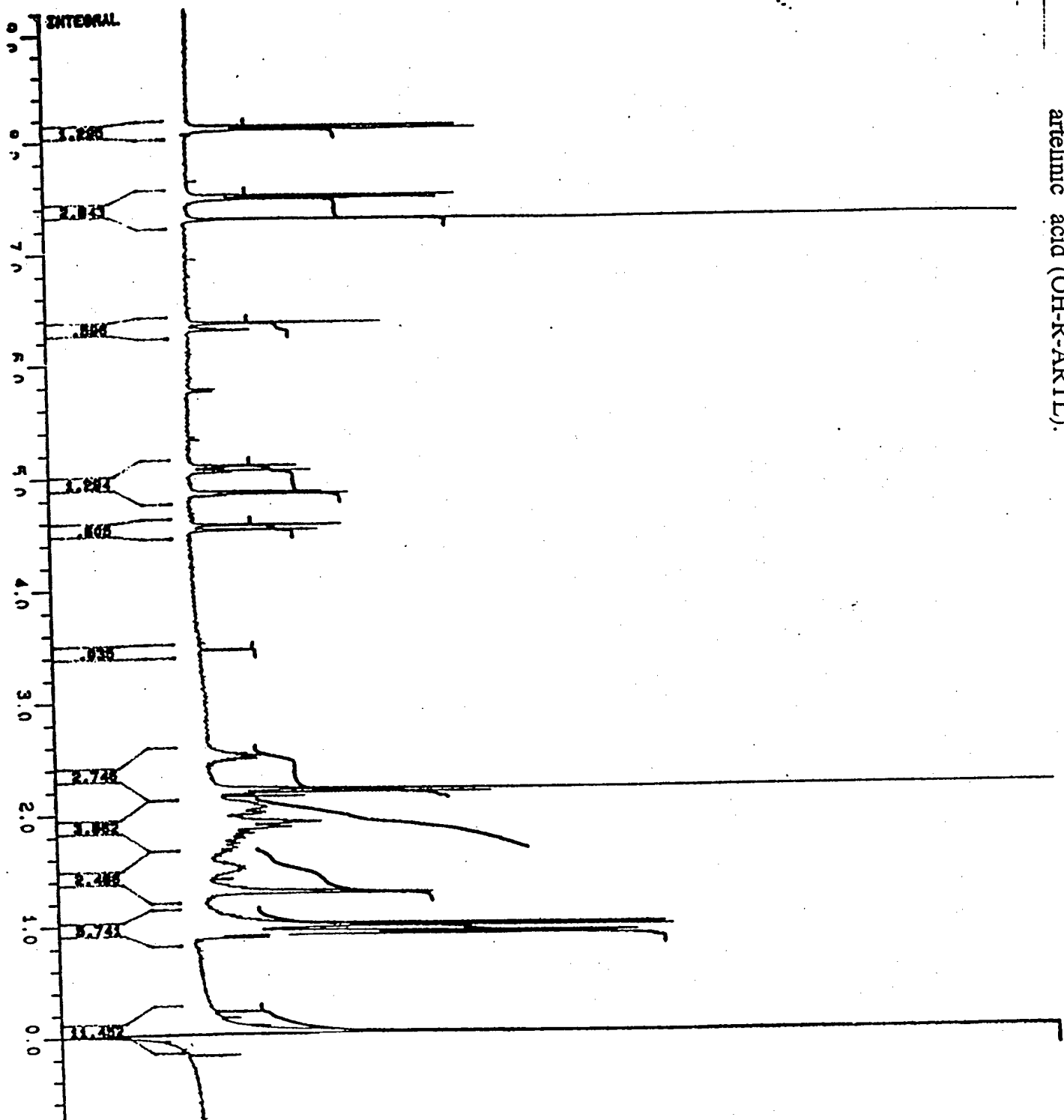
Figure 41. (a) Thermospray mass spectrum and (b) Negative ion mass spectrum of hydroxylated rearranged artelinic acid (OH-R-ARTL).

Figure 42. Proposed fragmentation pattern for the thermospray mass spectrum of hydroxylated rearranged artelinic acid (OH-R-ARTL).
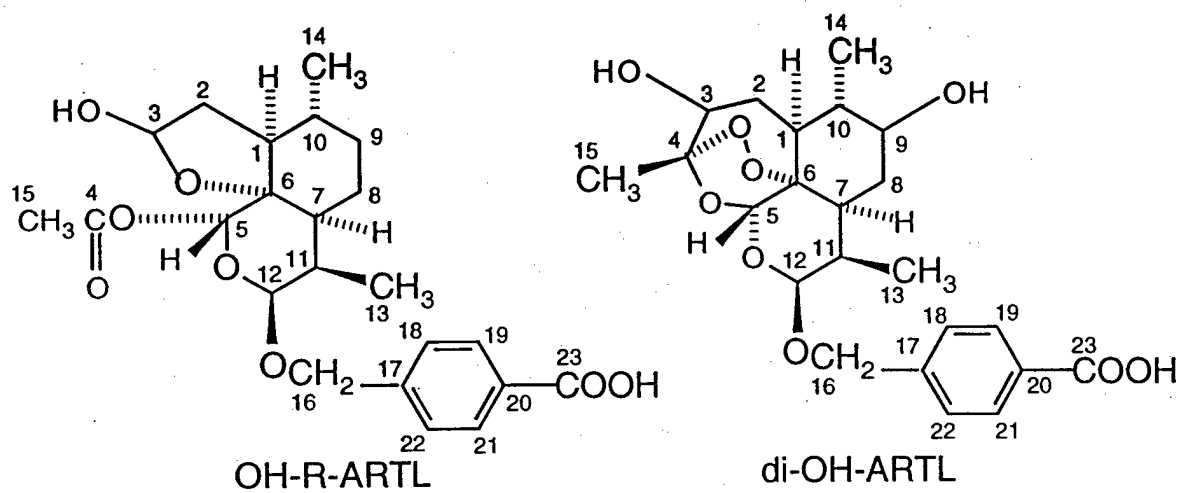
FIGURE 43. Structures of hydroxylated artelinic acid derivatives
formed by the reaction of artelinic acid with the iron(II)
complexes of 8-hydroxyquinolines.

Figure 44. 1H-nmr spectrum of dihyroxylated artelinic acid derivative (di-OH-ARTL).

Figure 45. 13C-nmr spectrum of dihydroxylated artelinic acid derivative (di-OH-ARTL).

PPM

RAMI1-C13

144.136

130.255
126.976

103.937
101.772

87.501
77.318
77.100
76.893
76.411
73.016
72.111
68.953

50.910
49.200
44.495

34.822
31.930
30.862
28.153
24.465
20.115
17.138
13.037

-.118
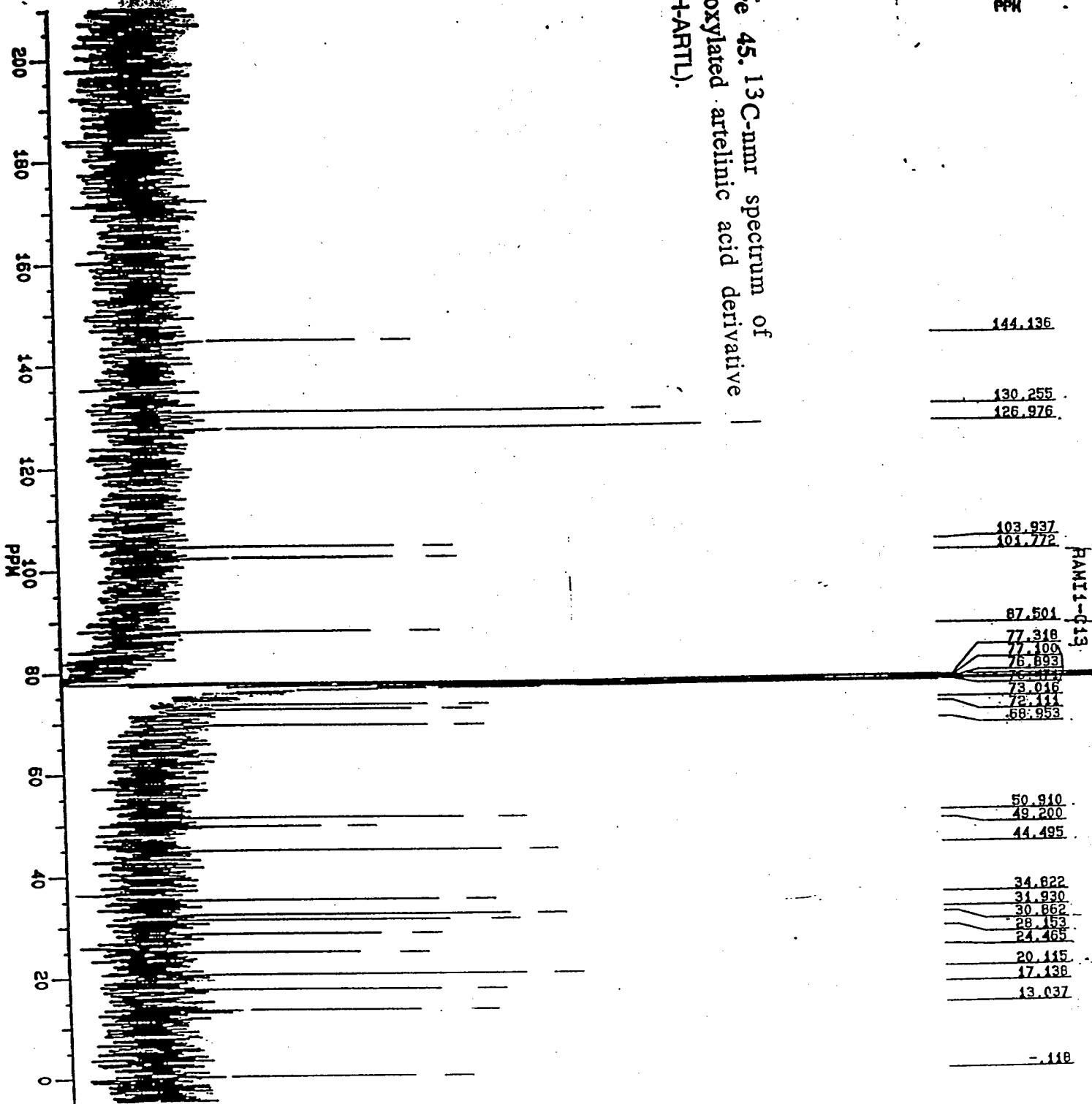
RYM10S.101
AU PROG:
XO2.AU
DATE 26-5-98

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TD   32768
SW   18518.519
HZ/PT   1.130

PW   0.0
RD   0.0
DP   20H DO

FW   23200
O2   4588.000
NS   12800
RG   800
AQ   .885
TE   297

LB   1.000
GB   .100
CX   20.00
CY   10.00
F1   210.005P
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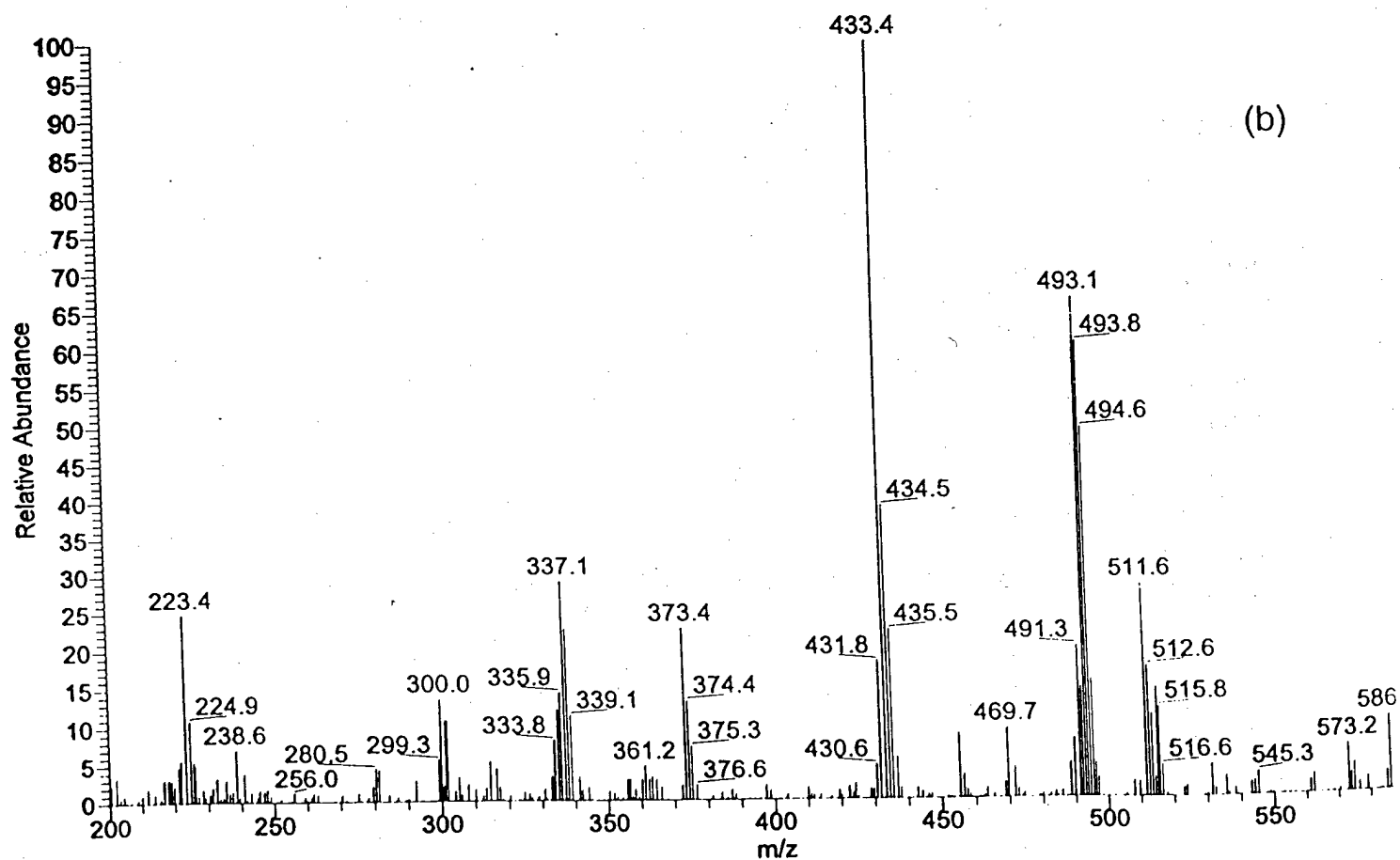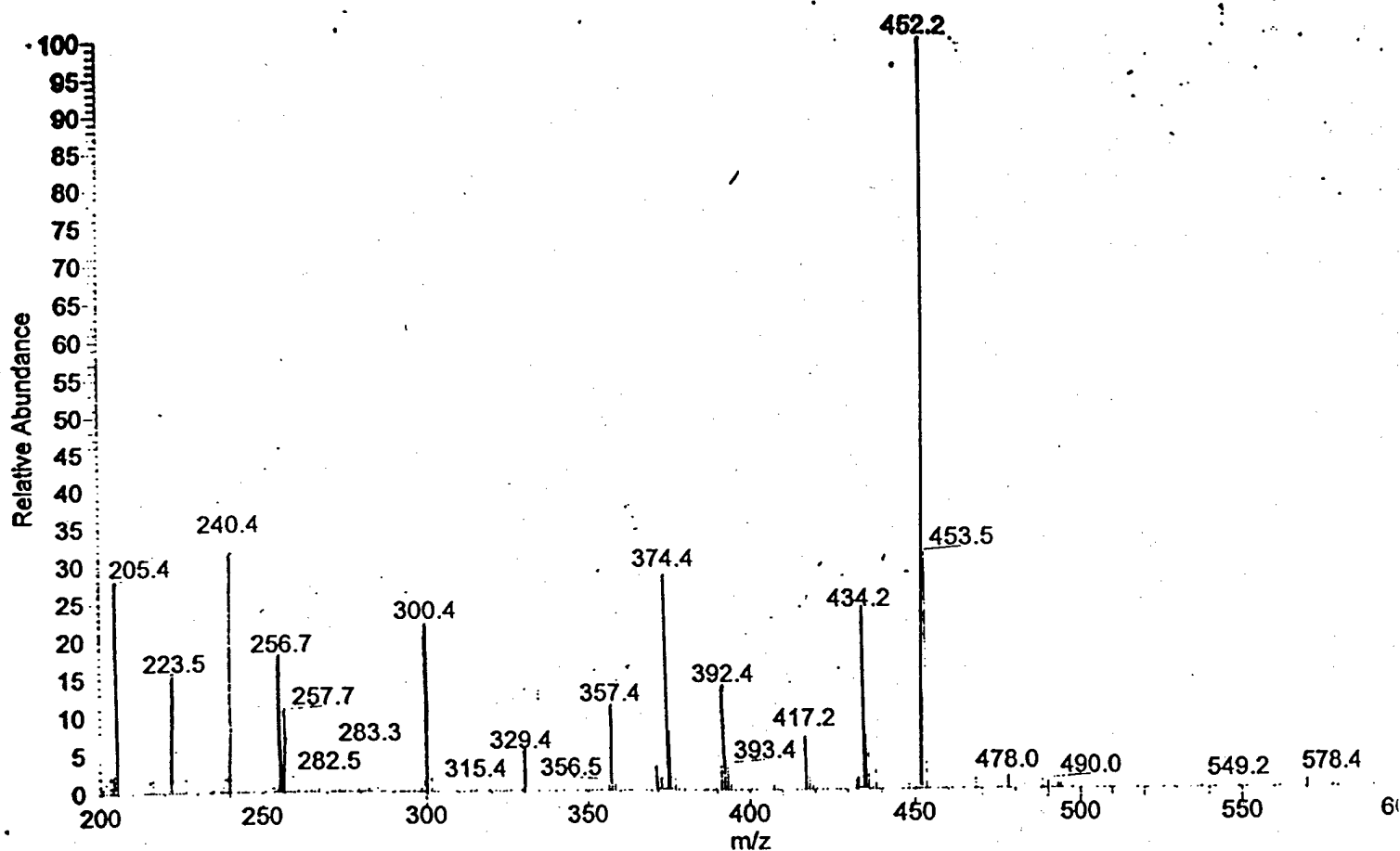HZ/CM 811.259
PPM/CM 10.750
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BRUKER
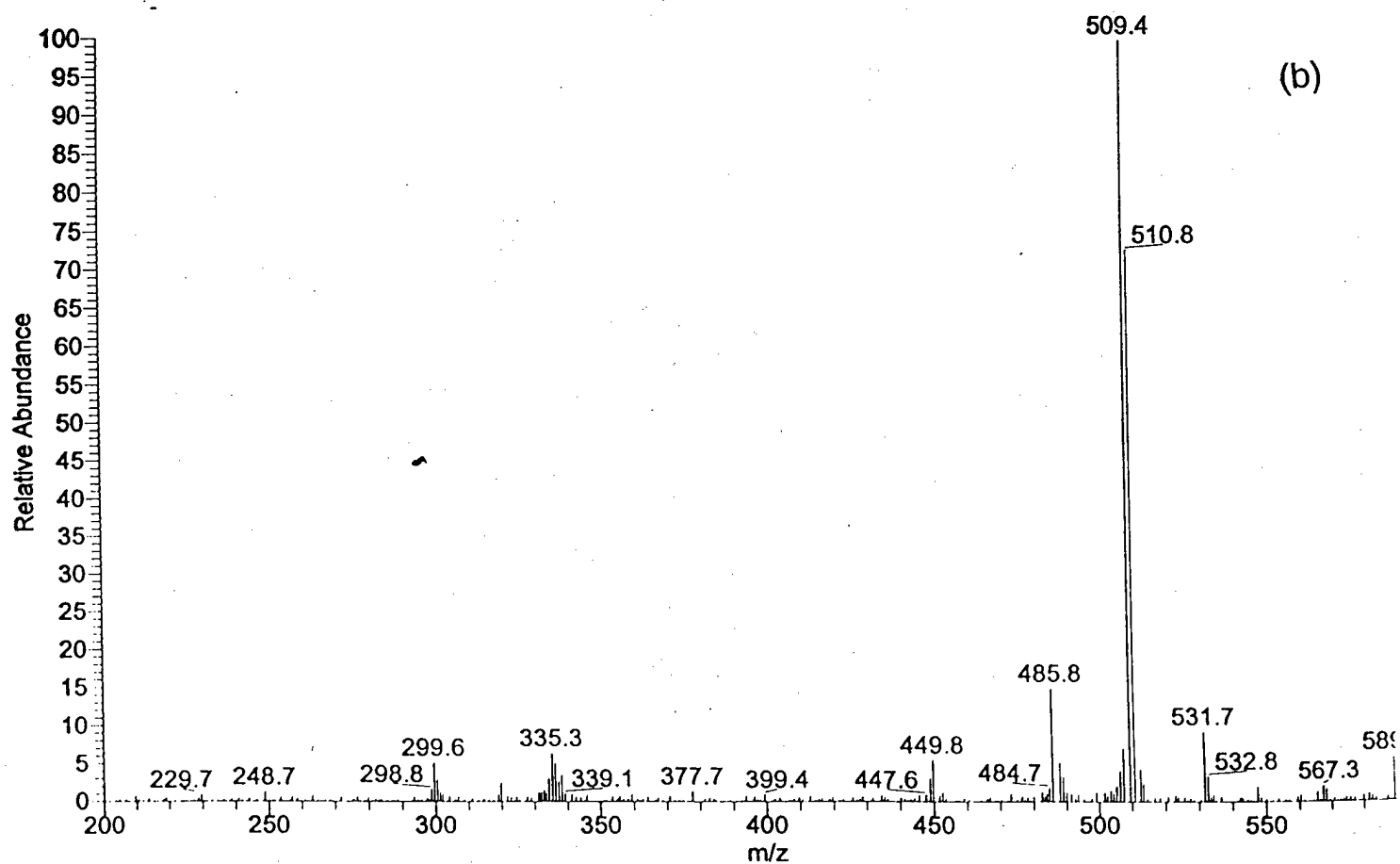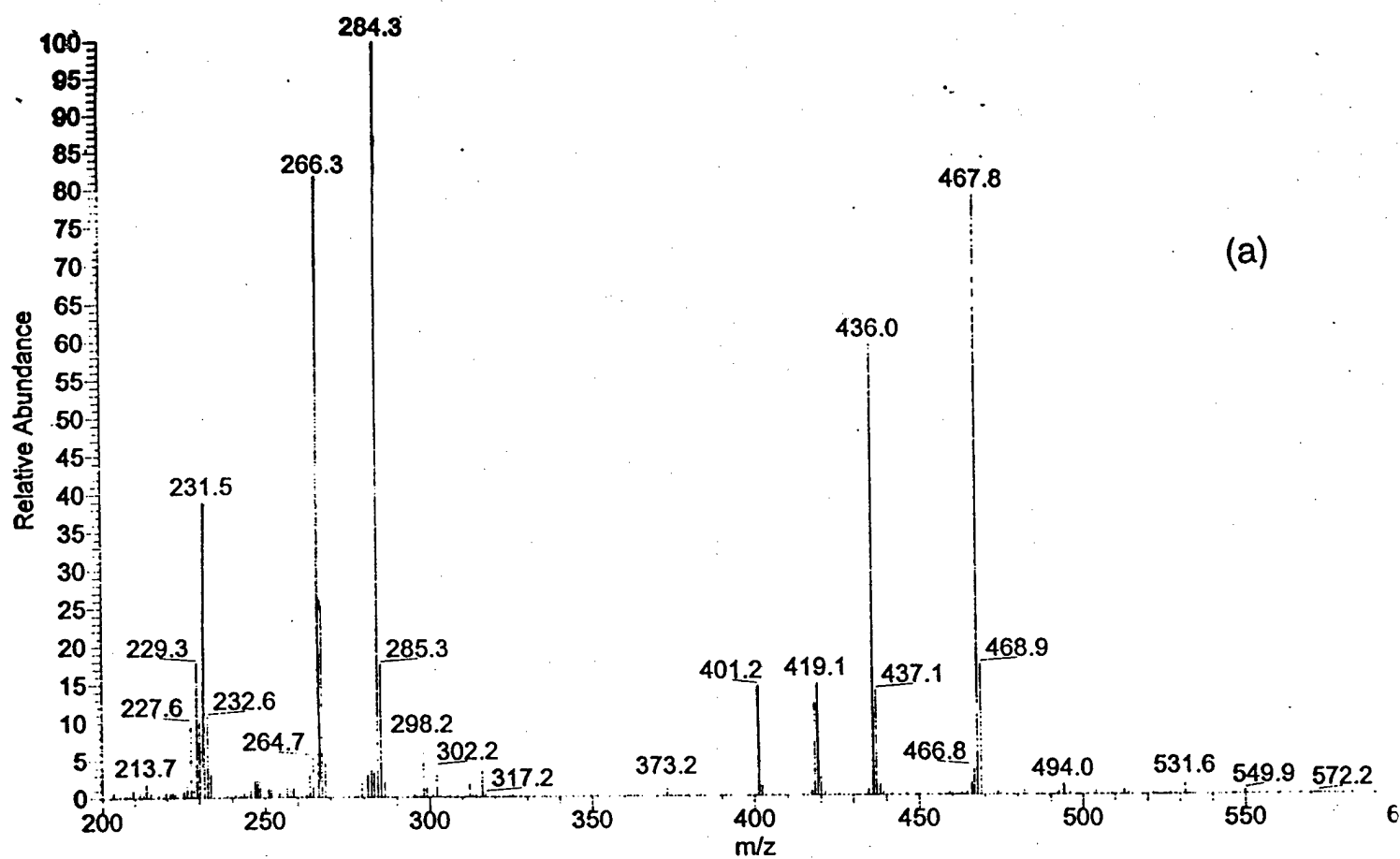
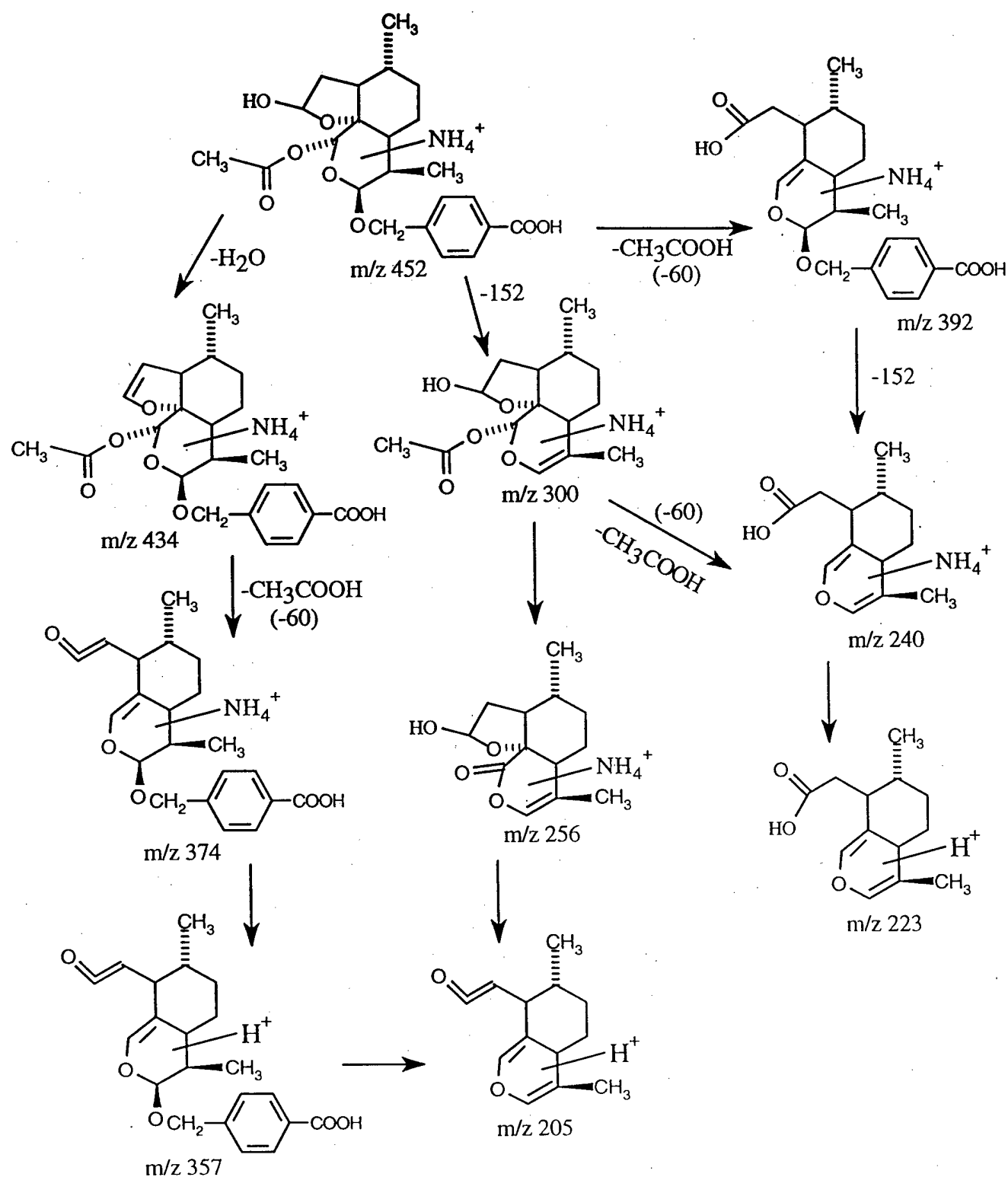Figure 46. (a) Thermospray mass spectrum and (b) Negative ion mass spectrum of dihydroxylated artelinic acid derivative (di-OH-ARTL).
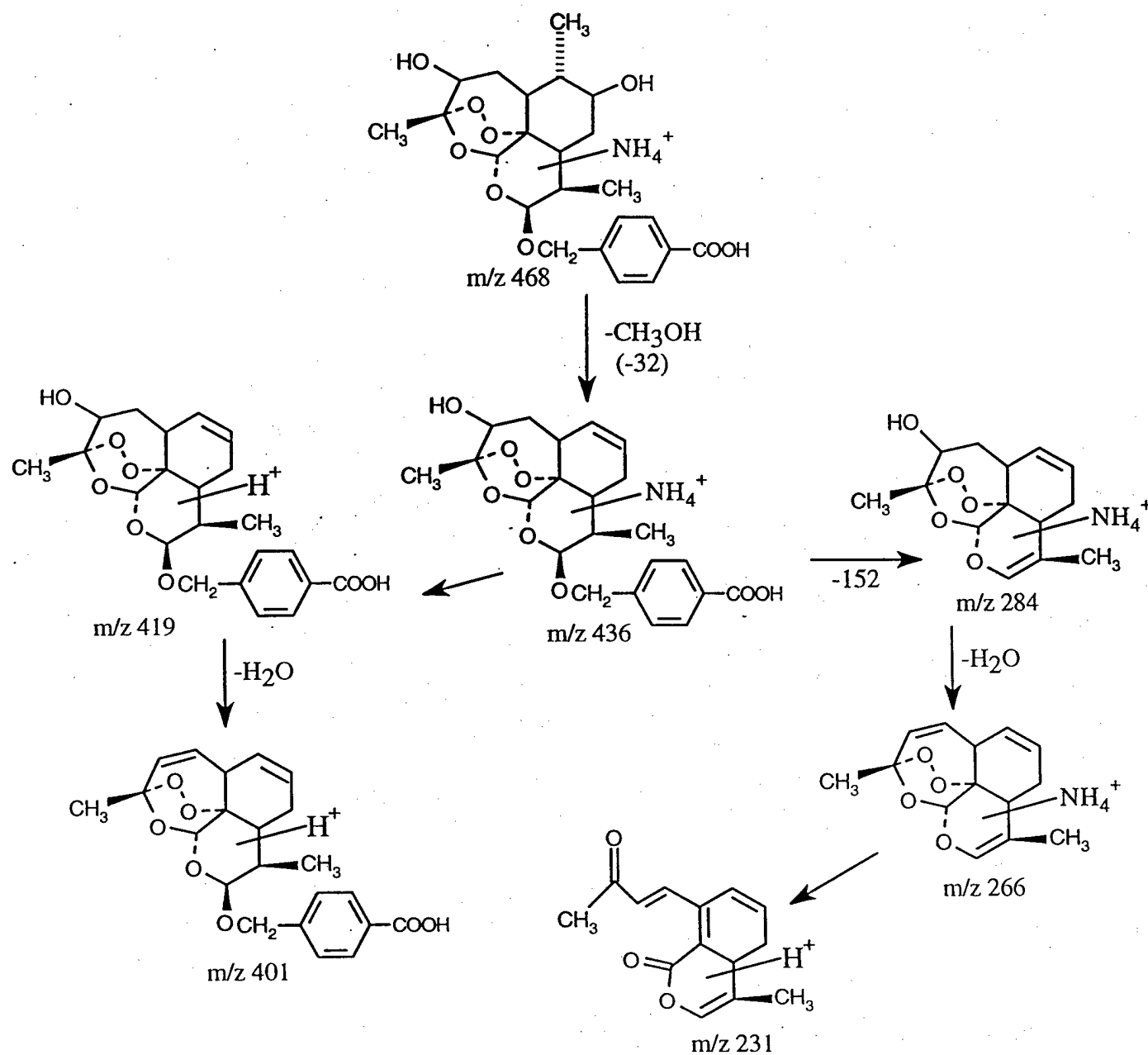
Figure 47. Proposed fragmentation pattern for the thermospray mass spectrum of dihydroxyartelinic acid (di-OH-ARTL)
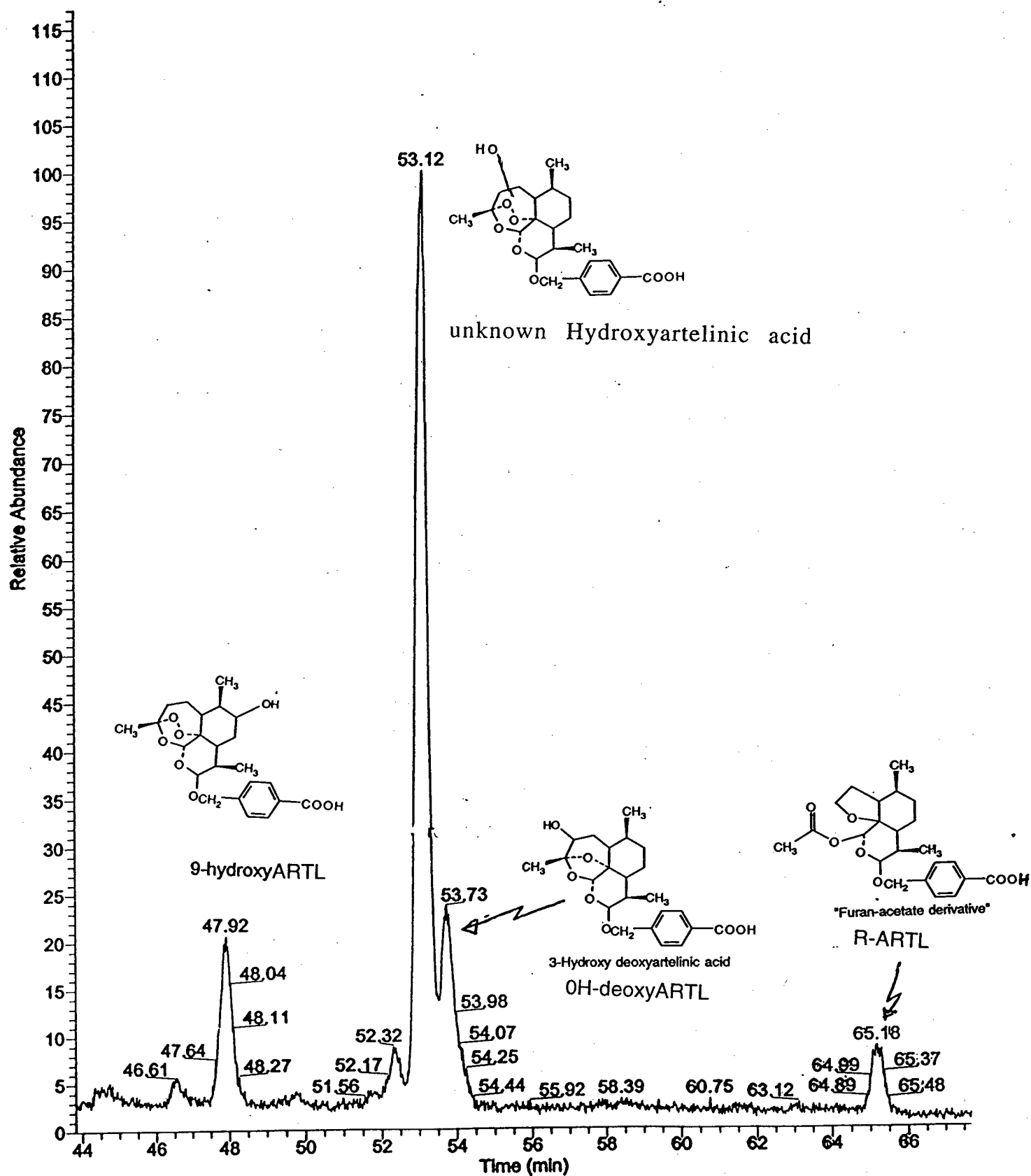
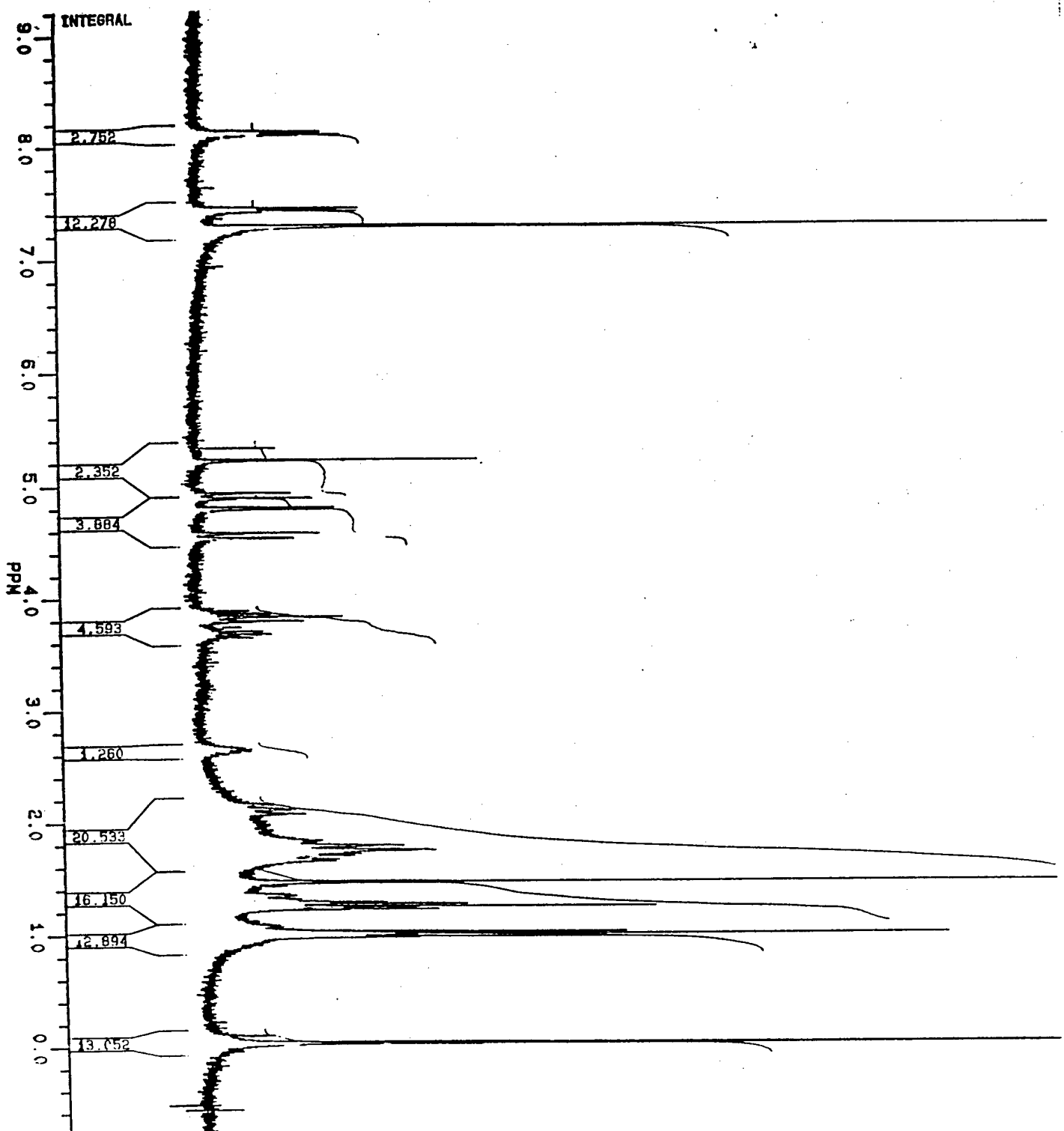Figure 48. Total ion chromatogram of compounds formed by the incubation of artelinic acid with liver microsomes.

APPENDIX 1

Biomimetic Metabolism of Artelinic Acid by Chemical Cytochrome P-450 Model Systems

Olutosin R. Idowu,[1,2] Ai J. Lin,[1] James M. Grace,[1] and James O. Peggins[1]

Purpose. To study the reaction of artelinic acid with chemical model systems of cytochrome P-450 as a means of obtaining authentic samples of the putative metabolites necessary for identification of the mammalian metabolites of artelinic acid.

Methods. Artelinic acid was reacted with different organic complexes of iron(II). The reaction products were isolated and characterized by NMR and thermospray mass spectroscopy.

Results. Five compounds which are putative metabolites of artelinic acid were isolated from these reactions and unambiguously identified, while the identity of two other compounds await final confirmation.

Conclusions. Standards of possible metabolites of artelinic acid can be produced by the reaction of the compound with ferrous complexes that may simulate cytochrome P-450 catalyzed metabolism of xenobiotics. This approach may provide a simple and versatile method for the formation of metabolites of artemisinin compounds which is more advantageous than previous approaches with fungal-based systems.

KEY WORDS: antimalarial; artelinic acid; metabolites; chemical simulation of metabolism.

INTRODUCTION

Qinghaosu (QHS) (also known as artemisinin), a sesquiterpene lactone with an endoperoxide linkage, is the novel, clinically active antimalarial principle isolated from the Chinese medicinal herb, Qinghao or *Artemisia annua* L (1,2).

The development of more potent semi-synthetic derivatives of QHS has focused on the reduction of QHS to the lactol, dihydroqinghaosu (or dihydroartemisinin; Figure 1, DQHS), and subsequent preparation of its ether (or ester) derivatives.

Artelinic acid (Figure 1, compound [1]), the 4-carboxybenzyl ether of DQHS, was developed in this institute (3). A study of the *in vitro* metabolism of artelinic acid has been initiated, towards the goal of developing artelinic acid as an oral treatment for uncomplicated malaria caused by multidrug resistant *P. falciparum*.

The major obstacle in metabolism studies of artemisinin analogues is the non-availability of standards for putative metabolites. This problem arises because total synthesis of the metabolites involves tedious and laborious steps. So far the only known approach to making authentic standards of the metabolites of an artemisinin analogue is through fermentation of the compound with fungi (4–8). However, a limitation of the fungal fermentation method is that it is difficult to predict

a microorganism that will closely mimic the mammalian metabolism of the compound. Reported data have also shown that fungal metabolism of these compounds does not correlate well with the mammalian metabolism. (4).

We have recently sought a more efficient and less time-consuming method for making the metabolites of the artemisinin compounds through the reaction of the compounds with chemical model systems of cytochrome P-450. The present work describes the biomimetic metabolism of artelinic acid by chemical models of cytochrome P-450 and the isolation and identification of putative metabolites of artelinic acid.

MATERIALS AND METHODS

Chemicals

All reagents and chemicals were obtained from Aldrich Chemical Co (Milwakee, USA) except artelinic acid and sodium artelinate which were from the inventory of the Walter Reed Army Institute of Research, Division of Experimental Therapeutics.

Instrumentation

Isolation of Reaction Products

Preparative HPLC was performed on a Waters liquid chromatography system consisting of two Waters model 510 solvent delivery units, a U6K injector and a Waters model 440 UV detector set at 254 nm. A Waters μ Bondapak C_{18} preparative column (7.8 mm × 300 mm; 10 mm) was used with a mobile phase consisting of 0.1M ammonium acetate buffer (pH 4.5) and acetonitrile. Separation was done using either a stepwise gradient of 70:30 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 56 min, then changed to 50:50, and held at this ratio for a further 40 min (flow rate 2.0 ml/min); or a gradient of 80:20 (v/v) 0.1M ammonium acetate:acetonitrile maintained for 40 min, changed to 70:30 (v/v) for 46 min, and then stepped to 50:50 and held for a further 30 min. Acetonitrile was removed from the collected fractions under a stream of nitrogen and the fractions were extracted with ethyl acetate (3 ml) by shaking on a vortex mixer for 2 min. The ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated under a stream of nitrogen. The residues were crystallized from a mixture of diethyl ether and hexane. The isolated products were again re-analysed by HPLC-MS as described below, to establish their purity.

The reaction products may also be separated by repeated preparative thin layer chromatography on 10 × 20 cm preparative silica gel GF plates, with dichloromethane:ethyl acetate (50/50, v/v) as solvent. Detection was by UV light, with the compounds showing up as dark bands on the fluorescent background. The bands were separated and extracted with 20% methanol in ethyl acetate, the solvent removed, and the residue crystallized from ether-hexane.

Characterization of Reaction Products

Mass spectrometric identification of the reaction products was performed using a HPLC-MS system consisting of a Hew-

[1] Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100.
[2] To whom correspondence should be addressed. (e-mail: Dr_Remi_Idowu@wrsmtp-ccmail.army.mil)

of the material being obtained from the ether extract of the precipitate. For initial purification, the oil was chromatographed on preparative TLC plates with ethyl acetate:dichloromethane (30:70, v/v) as solvent. A resinous material remained at the origin and the main band (rf about 0.3) was scraped and extracted with a 20:80 (v/v) methanol:ethyl acetate mixture yielding a pale yellow solid. The solid was fractionated by preparative HPLC.

The above procedure was repeated, using 740 mg (2.4 mmol) of glutathione or 500 mg (3.24 mmol) of thiosalicylic acid instead of cysteine.

Reaction of Artelinic Acid with Iron(II) Sulphate and Thiosalicylic Acid

Iron(II) sulphate (831.4 mg; 2.99 mmol) was dissolved in 50 ml of water and a solution of 922.2 mg (5.98 mmol) of thiosalicylic acid in 50 ml of acetone was added followed by 500 mg (1.2 mmol) of artelinic acid The mixture was stirred at room temperature for 18 h, during which time a beige-colored precipitate appeared. The mixture was centrifuged (3000 rpm, 10 min) and the supernatant (pH 3) was extracted with 50 ml of ethyl acetate. The extract was dried over anhydrous sodium sulphate and evaporated to obtain an oil. The oil was purified by preparative TLC. Three bands were formed of which the middle band (5.5–10.6 cm) was the main product. The material in the main band was recovered and purified by preparative HPLC.

Reaction of Artelinic Acid with Iron(II) Chloride and 8-Hydroxyquinoline (Oxine)

Iron(II) chloride tetrahydrate (475.6 mg: 2.392 mmol) was dissolved in about 10 ml of water and a solution of 694.5 mg (4.78 mmol) of 8-hydroxyquinoline (oxine) in 10 ml of acetone was added. A dark blue mixture formed immediately. Artelinic acid (200 mg; 0.479 mmol) was added and the mixture stirred for 16 h. The mixture was centrifuged (3000 rpm for 15 min), the supernatant separated and dried with a stream of nitrogen. The resulting residue was extracted with 40 ml of ethyl acetate. The ethyl acetate extract was washed successively with 2 × 20 ml of 0.05 M hydrochloric acid, 20 ml of distilled water, dried over anhydrous sodium sulphate, filtered through fluted filter paper and evaporated to dryness. The oily residue obtained was purified by preparative HPLC.

RESULTS AND DISCUSSION

Identification and Characterization of Compounds Formed

Extracts of reaction mixtures were initially examined by LC-MS to establish the presence of new compounds before chromatographic fractionation of the extracts was attempted. The total ion chromatograms of the crude products of the reaction of artelinic acid with the different reagents are shown in Figures 2 to 5. Chromatograms obtained for the products of the reaction of artelinic acid with hemin and thiosalicylic acid are also similar to those in Figure 3, except for the absence of peak [5].

The structures of the five compounds isolated and characterized after the reaction of artelinic acid with the above model
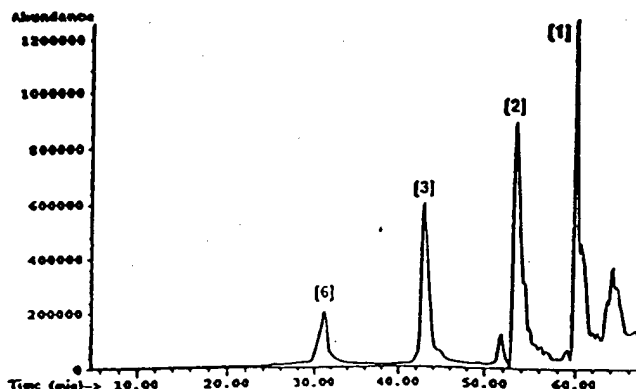

Fig. 2. Total ion chromatogram of the crude products of the reaction of artelinic acid with the Udenfriend system.

systems of cytochrome P-450 are shown in Figure 1. The identity of two other compounds (Figure 1, [5] and [8]) suspected to be formed by these reaction requires final confirmation. Of the compounds identified and characterized, the previously unreported ones are rearranged artelinic acid [2], 3α-hydroxy deoxyartelinic acid [3], and deoxy artelinic acid [4]. The ^{1}H- and ^{13}C-nmr assignments for these compounds are based on the published NMR data for analogous compounds obtained by the microbial metabolism of arteether (6,8) and artemisinin thermodecomposition products (9).

The relevant nmr and mass spectra data for artelinic acid (Figure 1, [1]) are provided for comparison: ^{13}C-nmr, see Table I. ^{1}H-nmr: 0.95 (3H, d, J = 6.0, Me-14), 0.98 (3H, d, J = 7.4, Me-13), 1.46 (3H, s, Me-15), 2.72 (1H, m, H-11), 2.03 (1H, m, H-3β), 2.40 (1H, d, d, d, J = 3.9, 4.07, 3.9, H-3α), 2.72 (1H, m, H-11), 4.61 (1H, d, J = 13.3, H-16β), 4.94 (1H, d, J = 3.4, H-12), 4.99 (1H, d, J = 13.3, H-16α), 5.47 (1H, s, H-5), 7.43 (2H, d, J = 8.2, H-18, H-22), 8.10 (2H, d, J = 8.2, H-19, H-21). Thermospray mass spectrum (m/z, relative intensity (%): 436, 3.2%; 401, 5.2%; 373, 4.0%; 359, 3.1%; 355, 3.0%; 331, 15.2%; 284, 3.1%; 249, 3.8%; 221, 100%.) In all the mass spectra presented, the highest m/z values are for the quasi-molecular $[M + NH_4]^+$ ion. The thermospray mass spectra of artelinic acid and related compounds formed in these reactions are characterized by fragmentation of the carboxybenzyl group (as carboxybenzyl alcohol, $OHCH_2C_6H_4COOH$; 152
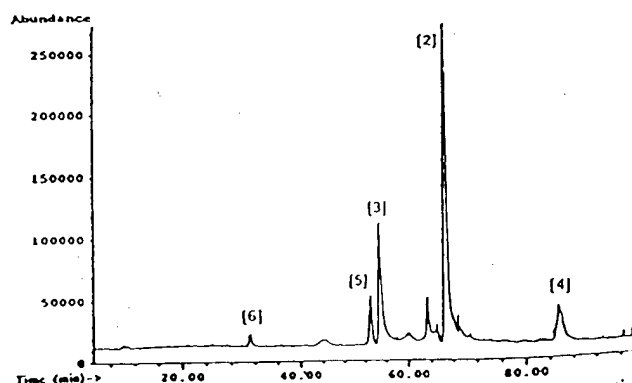

Fig. 3. Total ion chromatogram of the crude products of the reaction of artelinic acid with hemin-cysteine.

420, 0.4%; 357, 1.1%; 268, 16.5%; 252, 16.7%; 251, 100%; 233, 45.5%; 223, 2.8%; 215, 22.4%; 205, 19.6%.) The ¹H-nmr and ¹³C-nmr spectra of compound [4] are also consistent with those of deoxy arteether (5). The best yield of 23 mg (percent yield, 9%) of deoxyartelinic acid was obtained from the reaction system containing hemin and glutathione.

A hydroxyartelinic acid (Figure 1, [5]), with the position and stereochemistry of the hydroxy group as yet to be confirmed, is suspected to be one of the products of the reaction of artelinic acid with the hemin-based reagents and with the iron(II)-oxine reagent. The ¹H-nmr spectrum of this compound exhibits a quadruplet centered at δ 3.55 (5,6,8). The ¹H-nmr spectrum also displayed the same aromatic proton resonances as artelinic acid, indicating that the hydroxy group was not introduced onto the aromatic ring of artelinic acid. Thermospray mass spectrum (m/z, relative intensity (%): 452, 0.1%; 435, 0.2%; 392, 1.6%; 375, 3.4%; 357, 2.4%; 300, 35.7%; 283, 9.5%; 265, 18.1%; 240, 22.1%; 237, 13.5%; 224, 14.1%; 223, 100%; 222, 34.1%; 221, 10.1%; 195, 2.7%; 179, 7.3%; 177, 13.0%.) The thermospray mass spectrum is readily rationalized as that of a hydroxylated artelinic, with the formation of the ions involving the fragmentation of the 4-carboxybenzyl side-chain and thermal rearrangement of the compound during mass spectrometry, as experienced for artelinic acid itself.

3α-Hydroxydeoxydihydroqinghaosu (3α-OH-deoxy DQHS) (Figure 1, [6]) formed from artelinic acid was identified by comparison of its HPLC retention time, ¹H-nmr and mass spectra with those of an authentic standard. Thermospray mass spectrum (m/z, relative intensity (%): 302, 7.3%; 285, 11%; 284, 52.1%; 267, 52.9%; 266, 26.1%; 249, 100%; 239, 52.9%; 231, 17.9%; 221, 5.8%; 207, 6.8%.). 3α-OH-deoxyDQHS is a putative metabolite of artelinic acid and has also been reported to be a microbial metabolite of arteether (5).

Deoxydihydroqinghaosu (deoxyDQHS) (Figure 1, [7]) also was identified by comparison of its HPLC retention time and mass spectrum with those of an authentic standard. Thermospray mass spectrum (m/z, relative intensity (%): 286, 3.2%; 268, 16.2%; 252, 16.8%; 251, 100%; 233, 35.0%; 223, 59.9%; 215, 2.5%; 205, 3.3%.)

Rearranged dihydroqinghaosu (Figure 1, [8]) is suspected to be one of the compounds formed by the respective reaction of artelinic acid with the Udenfriend and the iron(II)-oxine systems on the basis of the similarity of its thermospray mass spectrum to that of rearranged artelinic acid. Thermospray mass spectrum of rearranged dihydroqinghaosu (m/z, relative intensity (%): 302, 4.4%; 284, 1.6%; 267, 2.0%; 242, 74.7%; 225, 19.3%; 224, 13.1%; 207, 17.2%; 206, 100%; 195, 0.8%.)

LC-MS of the extract of the Udenfriend system showed that DQHS was formed in this system.

4-(Hydroxymethyl)benzoic acid identified by its ¹H-nmr spectrum, was recovered by TLC from the reaction of artelinic acid with the Udenfriend, iron(II)-thiosalicylic and iron(II)-oxine systems.

No compound resulting from the hydroxylation of the aromatic ring of artelinic acid was observed in this study.

Simulation of the Metabolism of Artelinic Acid

Although many chemical models of P-450 have been studied, only very simple compounds such as cyclohexane, styrene, adamantane, aniline, toluidine, etc have been used as substrates.

There have been only a few reports on the application of chemical models of the P-450 enzymes to study the metabolism of actual drugs. We studied the chemical simulation of the metabolism of artelinic acid as a means of obtaining milligram quantities of the likely metabolites of the compound.

From previous reports on the metabolism of artemisinin and arteether (4–8), the expected P-450 catalyzed metabolic reactions of artelinic acid are rearrangement and/or deoxygenation of the endoperoxide group, C-hydroxylation at the 1- or 2- or 3- or 9- or 14-position, and removal of the 4-carboxybenzyl side-chain.

The earliest of the chemical model systems of cytochrome P-450, which has come to be known as the Udenfriend system, consists of a mixture of ferrous ion, ascorbic acid, EDTA, and molecular oxygen (10). The Udenfriend system brought about the biomimetic rearrangement of artelinic acid to compound [2] and 3α-hydroxydeoxyartelinic acid [3]. This system also causes the removal of the 4-carboxybenzyl side-chain of artelinic acid. Deoxygenation of artelinic acid to deoxyartelinic acid was not observed with this reagent.

The simulation of cytochrome P-450 by the combination of hemin and thiol-containing compounds such as cysteine and glutathione was extensively studied by Sakurai and co-workers (11). The present work is the first attempt to apply these systems to a study of the metabolism of a drug. When the reaction of artelinic acid and the hemin-thiol reagents was carried out in a purely aqueous medium most of the artelinic acid was recovered unchanged, with only traces of [2] and deoxyartelinic acid [4] being detected by LC-MS. When the reaction was carried out in aqueous acetone, a virtually complete transformation of artelinic acid was observed, with the unchanged compound being only detectable by LC-MS. The hemin-thiol systems brought about the deoxygenation of artelinic acid to deoxyartelinic acid. These systems were virtually ineffective in the dealkylation of artelinic acid. Thus, neither DQHS nor deoxyDQHS was formed, and only traces of 3α-OH-deoxyDQHS were detected, in the hemin-thiol systems. A compound suspected to be a hydroxyartelinic acid was also formed in the hemin-thiol systems.

The combination of iron(II) with thiosalicylic acid is one of the early variations on the Udenfriend system, and this reagent was found to hydroxylate simple aromatic and aliphatic hydrocarbons (12). The iron(II)-thiosalicylic acid system brings about the biomimetic rearrangement, deoxygenation and dealkylation of artelinic acid. Also, a compound (Figure 4, [U]) suspected to be a hydroxylated rearranged artelinic acid was detected by LC-MS from the iron(II) thiosalicylic acid system.

The simulation of cytochrome P-450 drug metabolizing activity by the combination of iron(II) and 8-hydroxyquinoline (oxine) has not been reported previously. The iron(II)-oxine system caused the rearrangement of artelinic acid into [2] and 3α-hydroxydeoxyartelinic acid [3], catalyzed its deoxygenation to deoxyartelinic acid [4] and possible hydroxylation to a hydroxyartelinic acid. In addition, this system is capable of effecting biomimetic dealkylation as evidenced in the formation of deoxyDQHS and the compound tentatively identified as rearranged DQHS.

CONCLUSIONS

Standards of metabolites of artelinic acid can be produced by the reaction of the compound with simple reaction systems

APPENDIX 2A

DATA SHEET FOR CHEMICAL COMPOUNDS

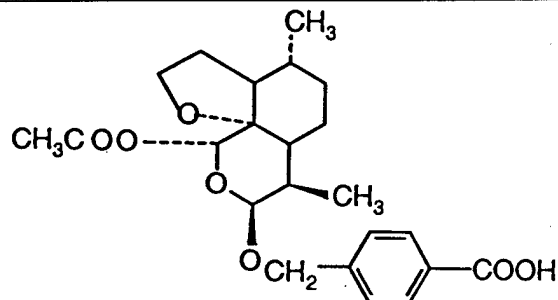|	
RECV: 17-OCT-96	SUBMITTER: DR. OLUTOSIN REMI IDOWU
	ET
SUB CODE: ARTL/M-1	WRAIR, WRAMC
WRNO : 280849	DIVISION OF EXPER. THERAPS.
BOTTLE #: BN81302	WASHINGTON, DC 20307
SUBMTR #: 0300	

CHEMNAME: ?

SALT: NONE

STRUCTURE:

FORMULA: C23H30O7
MOL WT :   418.5

APPEARANCE: WHT/POWDER

QTY: 10M   MILLIGRAMS

HYGROSCOPIC:	M.P.:
TEST SYS:	PGS: G
SHELF LOCATION: NEVER	

STABILITY:

SOLUBILITY:

WARNING: NONE KNOWN

Literature:

EQUATIONS INDICATING SYNTHETIC ROUTE:

STORAGE : COLD
REMARKS :

Commercially Discreet:	Contract#: DAMD17- Synth. under Gov't Support:	Gift:	Purchased:

APPENDIX 2B

DATA SHEET FOR CHEMICAL COMPOUNDS

RECV: 03-FEB-97	**SUBMITTER:** ET DR. OLUTOSIN REMI IDOWU
SUB CODE: ARTL/M-2	WRAIR, WRAMC
WRNO : 280987	DIVISION OF EXPER. THERAPS.
BOTTLE #: BN85499	WASHINGTON, DC 20307
SUBMTR #: 0300	

CHEMNAME: 3-ALPHA-HYDROXYDEOXYARTELINIC ACID

SALT: NONE

STRUCTURE:
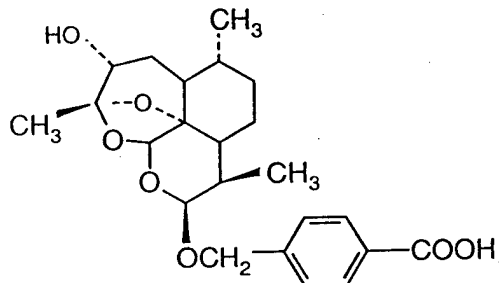
FORMULA: C23H30O7 **MOL WT :** 418.5	**STABILITY:**
	SOLUBILITY:
APPEARANCE: WHT/GRANULES	**WARNING:**
QTY: 3M MILLIGRAMS	**Literature:**
HYGROSCOPIC: | **M.P.:**	
TEST SYS: | **PGS:** S	
SHELF LOCATION: NEVER	

EQUATIONS INDICATING SYNTHETIC ROUTE:

STORAGE :

REMARKS :

Commercially Discreet:	Contract#: DAMD17- Synth. under Gov't Support:	Gift:	Purchased:

APPENDIX 2C

DATA SHEET FOR CHEMICAL COMPOUNDS

RECV: 03-FEB-97	**SUBMITTER:** DR. OLUTOSIN REMI IDOWU ET
SUB CODE: ARTL/M-3	WRAIR, WRAMC
WRNO : 280988	DIVISION OF EXPER. THERAPS.
BOTTLE #: BN85506	WASHINGTON, DC 20307
SUBMTR #: 0300	

CHEMNAME: DEOXYARTELINIC ACID

SALT: NONE

STRUCTURE:

FORMULA: C23H30O6	**STABILITY:**
MOL WT : 402.5	
	SOLUBILITY:
APPEARANCE: BEI/POWDER	
	WARNING:
QTY: 10M MILLIGRAMS	
	Literature:
HYGROSCOPIC: | **M.P.:**	
TEST SYS: | **PGS:** S	
SHELF LOCATION: NEVER	

EQUATIONS INDICATING SYNTHETIC ROUTE:

STORAGE :
REMARKS :

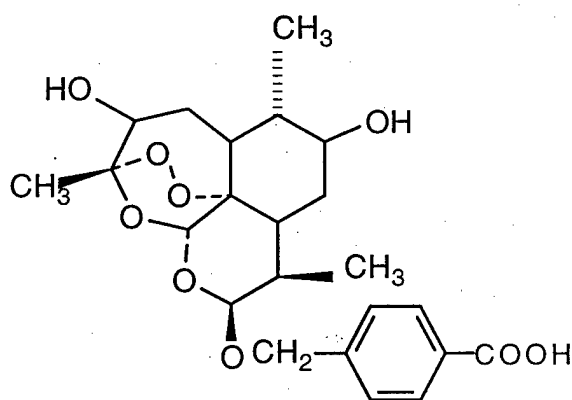| Commercially Discreet:	Contract#: DAMD17- Synth. under Gov't Support:	Gift:	Purchased:

APPENDIX 2D

Compounds recently submitted to the Walter Reed inventory:
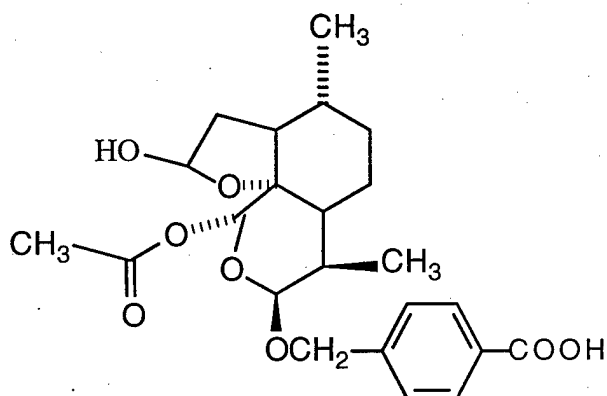
Rearranged Hydroxyartelinic acid (R-OH-ARTL)

Amount submitted : 10 mg

3, 9-Dihyroxyartrelinic acid (Di-OH-ARTL)

Amount submitted: 20 mg

In addition, 500 mg of rearranged artelinic (Appendix 2A) and 55 mg of 3-hydroxydeoxyartelinic acid (Appendix 2B) have been recently submitted to the Walter Reed inventory.